REMARKS

Claims 1-49 were pending in the application. Claims 1, 4, 8, 11, 15, 17-18, 21, 23, 25, 28, 42 and 48 have been amended and claims 7, 14, 20, 27, 38-39 and 46-47 have been canceled, without prejudice. Accordingly, upon entry of the amendments presented herein, claims 1-6, 8-13, 15-19, 21-26, 28-37 40-45 and 48-49 will remain pending in the application.

No new matter has been added. Support for the amendments to the claims can be found in the claims and throughout the specification as originally filed. In particular, support for the expression "low dose of 0.01 – 0.1 mg/kg of a TNFα inhibitor" can be found at least at page 19, first paragraph, and in particular at lines 11-13; in Table 2 at page 29; and in Figure 5.

Amendments to and cancellation of the claims should in no way be construed as an acquiescence to any of the Examiner's rejections and were done solely to expedite the prosecution of the application. Applicants reserve the right to pursue the claims as originally filed in this or a separate application(s).

Information Disclosure Statement

Responsive to the Examiner's request, Applicants provide herewith copies of references C1-D4 cited in the Information Disclosure filed on January 18, 2005.

Rejection of Claims 1-6, 8-13, 15-19, 21-26, 28-37, 40-45 and 49 Under 35 U.S.C. 112, Second Paragraph

The Examiner has maintained the rejection of claims 1-6, 8-13, 15-19, 21-26, and 28-31 and has further rejected claims 32-37, 40-45 and 49 under 35 U.S.C. § 112, second paragraph as being "indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention." In particular, the Examiner is of the opinion that these claims "are indefinite in the recitation of a 'low dose' therapy, because the metes and bounds of the claimed invention are unclear, so that one of ordinary skill in the art would not be reasonably apprised of the metes and bounds of the invention."

Applicants respectfully traverse the foregoing rejection on the grounds that claims 1-6, 8-13, 15-19, 21-26, 28-37, 40-45 and 49 particularly point out and distinctly claim the subject matter which Applicants regard as their invention, as required by 35 U.S.C. § 112, second paragraph. However, in the interest of expediting prosecution and in no way acquiescing to the

validity of the Examiner's rejection, the pending claims have been amended to specify that the "low dose" is a dose of ***0.01 – 0.1 mg/kg*** of a TNFα inhibitor. Accordingly, the foregoing rejection has been rendered moot and Applicants respectfully request that the rejection of claims 1-6, 8-13, 15-19, 21-26, 28-37, 40-45 and 49 under 35 U.S.C. § 112, second paragraph be reconsidered and withdrawn.

Rejection of Claims 1-4, 8-11, 15-17, 21-24, 28-29 and 31

Under 35 U.S.C. 112, First Paragraph, Enablement

The Examiner has maintained the rejection of claims 1-4, 8-11, 15-17, 21-24, 28-29 and 31 under 35 U.S.C. 112, first paragraph because, according to the Examer, "the specification, while being enabling for a TNFα inhibitor which is an anti-TNFα antibody, does not reasonably provide enablement for the full breadth of the genus of "TNFα inhibitors." In particular, the Examiner is of the opinion that

> [a]pplicant's arguments have been fully considered but have not been found convincing.
>
> Applicant argues that one of ordinary skilled in the art would recognize based on the teachings in the specification and the knowledge in the art at the time of filing that any compound which inhibits TNFα may be used in a low dosage therapy of the invention.
>
> This is not found persuasive, at least because the instant claims include in their breadth any TNFα inhibitors, including those which are currently not known in the art and for whichno "ordinary dosage has been established. Therefore, it is unpredictable whether a dose which is "substantially lower" than one that may be determined to be "ordinary" will also be effective.
>
> Applicant further argues that the specification discloses "numerous" examples of TNFα inhibitors to support a genus of inhibitors.
>
> This is not found persuasive, because although the specification discloses three examples of TNFα inhibitors, namely D2E7, Remicade and Embrel, they are disclosed to differ markedly in their effect on the microscopic signs of disease in a

mouse model of rheumatoid arthritis at a dose of e.g. 0.1 mg/kg (e.g. pages 29-30). In view of this variability, it remains unpredictable how effective other inhibitors of TNFα will be in alleviating microscopic or other symptoms of the disease, in model systems or in disease subjects.

Therefore, the rejection of record is maintained for the reasons of record, as it applies to the amended claims. The rejection of record is incorporated by reference herein, as if reiterated in full.

Applicants respectfully traverse this rejection for the following reasons.

The pending claims are directed to a method for treating a disorder in which TNFα activity is detrimental comprising administering to a subject an effective amount of a TNFα inhibitor in a ***low dose of 0.01-0.1mg/kg***, such that the disorder is treated. The pending claims are also directed to a low dose method to alleviate symptoms associated with a disorder in which TNFα activity is detrimental, comprising administering a ***low dose of 0.01-0.1mg/kg*** of a TNFα inhibitor to a subject suffering from said disorder, such that the symptoms are treated. The pending claims are further directed to a method of sequestering TNFα into complexes in a subject suffering from a disorder in which TNFα activity is detrimental, by administering a ***low dose of 0.01-0.1mg/kg*** of a TNFα inhibitor to the subject.

Section 112 does not require Applicants to describe every equivalent within the scope of the claims so long as the specification provides sufficient teaching for a person of skill in the art to identify additional equivalents ***without undue experimentation*** (In re Wands 8 USPQ2d 1400-1407, 1404 (CAFC, 1988)). The fact that some experimentation is required does not preclude enablement. See *e.g.*, In re Angstadt, 537 F.2d 498, 503. In fact, the Court of Appeals for the Federal Circuit has announced a test for enablement requiring that the general description set forth sufficient detailed guidance that one of ordinary skill in the art would have a reasonable expectation of success in carrying out the claimed invention. See *e.g.*, in re Wright, 999 F.2d 1557 (Fed. Cir. 1993). The fact that some experimentation may be required does not constitute a lack of enablement ***as long as the amount of experimentation is not unduly extensive***. Amgen Inc. v. Chugai Pharmaceutical Co., Ltd., 927 F.2d 1200, 1213 (CAFC 1991). Moreover, "as long as the specification discloses at least one method (emphasis added) for making and using the claimed invention that bears a reasonable correlation to the entire scope of the claim, then the

enablement requirement of §112 is satisfied." In re Fischer, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970).

Applicants respectfully submit that based on the teachings in Applicants' specification as well as the general knowledge in the art, one of skill in the art would be able to make and use the claimed invention using only routine experimentation.

Specifically, the instant specification teaches several examples of TNFα inhibitors which are suitable for use in the claimed methods, including etanercept, infliximab, human anti-TNFα monoclonal antibodies, CDP 571, and CDP 870 (see page 5, lines 11-14). The specification also contains working examples demonstrating the efficacy of three different TNFα inhibitors, including a chimeric antibody, a human antibody, and a fusion protein, in the claimed methods. As described at page 27, lines 20-26 of the specification, each of the three TNFα inhibitors was administered to mice at doses ranging from 0.01 mg/kg -10 mg/kg, including the doses of 0.01 mg/kg and 0.1 mg/kg, the lower and upper limit, respectively, of the claimed dosage range. The results demonstrate that mice treated with these various TNFα inhibitors show improvements in their symptoms, including a decrease in joint inflammation and joint vascularity, as well as cartilage and bone erosion. Thus, the examples provided in the specification demonstrate that the claimed low dose methods may be successfully practiced using various TNFα inhibitors.

Moreover, the specification provides sufficient teachings such that a person of skill in the art would be able to identify additional TNFα inhibitors suitable for use in the claimed methods without undue experimentation. The instant specification describes at least in Example 1, at pages 26-30, experiments which can be carried out in a mouse model to evaluate the efficacy of a particular TNFα inhibitor in alleviating arthritic symptoms at the claimed dosage range. In particular, Example 1b teaches evaluation of development of arthritis and Example 1(d) teaches microscopic analysis of vascularity, inflammation, cartilage and bone erosion to evaluate the efficacy of a TNFα inhibitor. One of skill in the art would recognize that these assays taught in the specification may be used to evaluate and identify other TNFα inhibitors useful in the claimed low dose methods. Thus, given the extensive guidelines and working examples provided by Applicants, combined with the high skill level in the art, testing of a given TNFα inhibitor for use in the subject invention would not constitute undue experimentation.

The Examiner has asserted that "although the specification discloses three examples of TNFα inhibitors, namely D2E7, Remicade and Embrel, they are disclosed to differ markedly in

their effect on the microscopic signs of disease in a mouse model of rheumatoid arthritis at a dose of e.g. 0.1 mg/kg." Applicants respectfully submit that although the three anti-TNFα inhibitors tested may have exhibited slightly varied responses, these inhibitors were effective in reducing inflammation, vascularity, cartilage erosion and bone erosion at low doses. For example, Remicade was effective in reducing vascularity, cartilage erosion and bone erosion at a dose falling within the claimed range (see Table 2 at page 29 of the specification). D2E7 was also effective in reducing inflammation, vascularity, cartilage erosion and bone erosion at a dose of falling within the claimed range (see Table 2 at page 29 of the specification).

In summary, Applicants respectfully submit that, in view of the ample teachings provided in the specification and the extensive knowledge available in the art, a person of ordinary skill in the art would be able to make and use the claimed methods using only routine experimentation. Accordingly, Applicants respectfully request that the rejection of claims 1-4, 7-11, 14-17, 20-24, 27-29 and 31 under 35 U.S.C. 112, first paragraph, for lack of enablement be reconsidered and withdrawn.

Rejection of Claims 1-4, 8-11, 15-17, 21-24, 28-29 and 31

Under 35 U.S.C. 112, First Paragraph, Written Description

The Examiner has maintained the rejection of claims 1-4, 8-11, 15-17, 21-24, 28-29 and 31 under 35 U.S.C. 112, first paragraph as "containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention." Specifically, the Examiner is of the opinion that "Applicant is not in possession of a method for treating a disorder by administering a 'TNFα inhibitor,' as generically recited in the instant claims" for the reasons of record.

Applicants respectfully traverse this rejection for the following reasons. Applicants respectfully submit that the instant specification discloses a sufficient number of examples of TNFα inhibitors to support a genus of TNFα inhibitors. It is a well established principle of U.S. Patent Law that "[a] specification may, within the meaning of 35 U.S.C., § 112, First Paragraph, contain a written description of a broadly written claimed invention ***without describing all species that claim encompasses***." Utter v. Hiraga, 845 F.2d 993, 6 USPQ2d

1709 (Fed. Cir. 1988). Moreover, "a 'representative number' is an ***inverse function of the skill and knowledge in the art***. Satisfactory disclosure of a 'representative number' depends on whether one of skill in the art would recognize that the Applicant was in possession of the ***necessary common attributes or features of the elements possessed by the members of the genus in view of the species disclosed***." (see MPEP 2163(II.A.3.a.ii).

As described above, the instant specification teaches examples of different types of TNFα inhibitors, *e.g.*, anti-TNFα antibodies and fusion proteins containing extracellular domains of a TNFα receptor, and provides working examples of three species of TNFα inhibitors, *e.g.*, etanercept, infliximab and D2E7. The specification further teaches that a TNFα inhibitor may be selected, for example, based on its ability to inhibit TNFα activity in a standard *in vitro* assay such as the L929 neutralization assay (see page 7, line 1 of specification). Further, the relevant skill and knowledge in the art was high at the time of the invention, such that an artisan could easily recognize if an agent inhibits TNFα activity using standard assays known in the art. Moreover, in addition to the specific TNFα inhibitors disclosed in the specification, various other TNFα inhibitors were well known in the art at the time the invention was filed (see, for example, TNFα inhibitors described in U.S. Patent No. 5,519,000, U.S. Patent No. 6,143,866, U.S. Patent No. 5,654,407 and U.S. Patent No. 6,177,077). Thus, in view of the teachings in the specification and high level of skill and knowledge in the art, it is Applicants' position that the instant specification provides sufficient written description so as to convey to those skilled in the art that, at the time the application was filed, Applicants were in possession of the claimed invention.

In view of the foregoing, Applicants respectfully request that the rejection of claims 1-4, 7-11, 14-17, 20-24, 27-29 and 31 under 35 U.S.C. 112, first paragraph, for lack of written description be reconsidered and withdrawn.

Rejection of Claims 1- 49 Under 35 U.S.C. 102(b)

The Examiner has maintained the rejection of claims 1-31 and has further rejected claims 32-49 under 35 U.S.C. 102(b) as lacking novelty in view of U.S. Patent No. 6,258,562 (Salfeld *et al.*; hereinafter "the '562 patent"). The Examiner relies on the '562 patent for teaching compositions and methods of use relating to human anti-TNFα antibodies, including the

antibody D2E7. In particular, the Examiner states that the '562 patent teaches "an effective dose of the antibody is 0.1-20 mg/kg."

Applicants respectfully traverse this rejection of claims 1-49 on the grounds that the '562 patent fails to teach or suggest each and every element of the claimed invention in accordance with 35 U.S.C. §102(b).

The pending claims are directed to a method for treating a disorder in which TNFα activity is detrimental comprising administering to a subject an effective amount of a TNFα inhibitor in a ***low dose of 0.01-0.1mg/kg***, such that the disorder is treated. The pending claims are also directed to a low dose method to alleviate symptoms associated with a disorder in which TNFα activity is detrimental, comprising administering a ***low dose of 0.01-0.1mg/kg*** of a TNFα inhibitor to a subject suffering from said disorder, such that the symptoms are treated. The pending claims are further directed to a method of sequestering TNFα into complexes in a subject suffering from a disorder in which TNFα activity is detrimental, by administering a ***low dose of 0.01-0.1mg/kg*** of a TNFα inhibitor to the subject.

Under 35 U.S.C. 102, for a prior art reference to anticipate a claimed invention, the prior art must teach *each and every element* of the claimed invention. *Lewmar Marine v. Barient*, 827 F.2d 744, 3 USPQ2d 1766 (Fed. Cir. 1987). Furthermore, "the identical invention must be shown in as complete detail as is contained in the...claim." *Richardson v. Suzuki Motor Co.*, 868 F.2d 1226, 1236, 9 USPQ2d 1913, 1920 (Fed. Cir. 1989).

The pending claims are directed to methods utilizing ***a low dose comprising 0.01-0.1 mg/kg*** of a TNFα inhibitor. As acknowledged by the Examiner, the '562 patent teaches that a therapeutically effective dose range for human anti-TNFα antibodies is 0.1-20 mg/kg. Moreover, the '562 patent teaches that a preferred dose for human anti-TNFα antibodies is 1-10 mg/kg (see column 26, lines 26-29). Accordingly, the '562 patent fails to teach or suggest methods utilizing a ***low dose of a TNFα inhibitor comprising 0.01-0.1 mg/kg***. In view of the foregoing, Applicants respectfully request that this rejection of claims 1-49 under 35 U.S.C. 102(b) as lacking novelty over the '562 patent be reconsidered and withdrawn.

Rejection of Claims 1- 49 Under 35 U.S.C. 102(e)

The Examiner has maintained the rejection of claims 1-31 and has further rejected claims 32-49 under 35 U.S.C. 102(e) as lacking novelty in view of U.S. Patent No. 6.509,015 (Salfeld

et al.; hereinafter "the '015 patent"). The Examiner relies on the '015 patent for teaching compositions and methods of use relating to human anti-TNFα antibodies, including the antibody D2E7. In particular, the Examiner relies on the '015 patent for teaching that "the range of therapeutically effective amount of the antibody of the invention is 0.1-20 mg/kg, a range overlapping with the one instantly claimed."

Applicants respectfully traverse this rejection on the grounds that the '015 Patent fails to teach or suggest each and every element of the claimed invention in accordance with 35 U.S.C. §102(e).

Similar to the '562 patent, the '015 patent fails to anticipate the claimed invention in that there is no teaching or suggestion in the '015 patent to use a low dose ***of 0.01-0.1 mg/kg*** of a TNFα inhibitor. Indeed, there is no teaching or suggestion in the '015 patent to consider any doses which appear less efficacious in standard assays than saturating doses. Thus, the '015 patent fails to teach or suggest each and every element of the pending claims and, accordingly, Applicants respectfully request that this rejection of claims 1-49 under §102(e) over the '015 patent be reconsidered and withdrawn.

Rejection of Claims 1- 49 Under Obviousness-Type Double Patenting

The Examiner has maintained the rejection of claims 1-31 and has further rejected claims 32-49 under the judicially created doctrine of obviousness-type double patenting as being unpatentable in view of claims 1-100 of U.S. Patent No. 6.509,015 (Salfeld *et al.*; hereinafter "the '015 patent"). The Examiner is of the opinion that "[a]lthough the conflicting claims are not identical, they are not patentably distinct from each other for the reasons set forth in the prior Office Action."

Applicants respectfully traverse the aforementioned obviousness-type double patenting rejection on the grounds that the claimed low dose methods would not have been obvious over the claims of the '015 patent.

A nonstatutory basis exists for a double patenting rejection when the claimed invention is an obvious variation of an invention in an issued patent (MPEP 804(B)(1)). Accordingly, any analysis employed in an obviousness-type double patenting rejection parallels the guidelines for analysis of a 35 U.S.C. 103 obviousness determination. *In re Braat*, 937 F.2d 589, 19 USPQ2d 1289 (Fed. Cir. 1991); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir.

1985). To establish a *prima facie* case of obviousness, it is necessary for the Examiner to present evidence, preferably in the form of some teaching, suggestion, incentive or inference in the applied references, or in the form of generally available knowledge, that one having ordinary skill in the art would have been ***motivated*** to make the claimed invention and would have had a reasonable ***expectation of success*** in making the claimed invention. Under section 103, "[b]oth the suggestion and the expectation of success must be founded in the prior art, not in applicant's disclosure" (*Amgen, Inc. v. Chugai Pharmaceutical Co., Ltd.* 927 F.2d 1200, 1207, 18 USPQ2d 1016 (Fed. Cir. 1991), quoting *In re Dow Chemical Co.,* 837 F.2d 469, 473, 5 USPQ2d 1529, 1531 (Fed Cir. 1988)).

The methods of the invention are unique in that they embody Applicants' unexpected discovery that low doses, *e.g.*, 0.01-0.1 mg/kg, of TNFα inhibitors can be effective in treating the claimed disorders and alleviating symptoms associated with these disorders. Applicants teach in the specification various benefits associated with administering low doses of the antibody, including improvement in cartilage erosion (see, for example, Table 2 at page 29 of the specification). Applicants also teach in the specification that low doses of TNFα inhibitor may be advantageous as they may decrease side effects and may decrease the frequency of administration associated with the normally prescribed dose (see, for example, page 7, lines 20-22 of the specification).

In contrast, the '015 patent provides general guidance with regard to normally prescribed dosing. The '015 patent fails to teach or suggest methods which use low doses of a TNFα inhibitor, let alone 0.01-0.1 mg/kg of a TNFα inhibitor. In particular, as acknowledged by the Examiner, the '015 patent teaches that a therapeutically effective dose range for human anti-TNFα antibodies is 0.1-20 mg/kg. Further, the '015 patent teaches that a preferred dosage for human anti-TNFα antibodies is 1-10 mg/kg (see column 26, lines 26-29). Thus, the '015 patent fails to teach or suggest methods which use a ***low dose of 0.01-0.1 mg/kg of a TNFα inhibitor***. Moreover, one of ordinary skill in the art would not have been motivated to arrive at the claimed invention, *i.e.*, to select the claimed dosage range of 0.01 to 0.1 mg/kg, based on the disclosure of the '015 patent (see M.P.E.P § 2144.08).

In view of the foregoing, it is evident that the teachings of the '015 patent fail to render the claimed invention obvious. Accordingly, Applicants respectfully request that the Examiner reconsider and withdraw this obviousness-type double patenting rejection of claims 1-49.

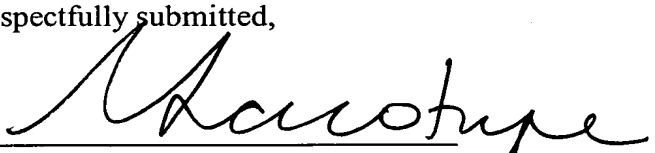
CONCLUSION

Reconsideration and allowance of all the pending claims is respectfully requested. If a telephone conversation with Applicants' Attorney would help expedite the prosecution of the above-identified application, the Examiner is urged to call the undersigned at (617) 227-7400.

In addition, Applicants include herewith authorization to charge fees associated with new claims and the extension of time with which to respond, to Deposit Account No. 12-0080, under Order No. BBI-190. The Director is also hereby authorized to charge any deficiency in the fees filed, asserted to be filed or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to Deposit Account No. 12-0080, under Order No. BBI-190.

Dated: **February 21, 2006**

Respectfully submitted,



Maria Laccotripe Zacharakis, Ph.D., J.D.
Registration No.: 56,266
Attorney for Applicants
LAHIVE & COCKFIELD, LLP
28 State Street
Boston, MA 02109
Tel. (617) 227-7400

2904

Production of neutralizing antibodies to tumor necrosis factor by human tumor-infiltrating B lymphocytes. Barbuto, J.A.M., Puni, C.J.A., Grimes, W.J.. Miller, T.P. and Hersh. E.M. Arizona Cancer Center, Tucson, AZ 85724

Infiltration of human tumors by lymphocytes is observed frequently and there is evidence for tumor-specificity of these cells. We have developed methodology to induce clonal expansion of tumor-infiltrating B lymphocytes (TIL-B) and to analyze the repertoire of their immunoglobulin (Ig) responses. Culture supematants of TIL-B expanded in the presence of feeder T cells protected actinomycin D-sensilized L929 murine fibroblast from tumor necrosis factor (TNF) cytotoxicily. These supematants were positive for TNF-specific Ig by ELISA. Anti-TNF B cell clones were recovered from TIL-B in ovarian cancer; colon cancer and in melanoma, with frequencies ranging from 1/26,000 to 1/11,000 cells. When B lymphocytes from normal donor peripheral blood (PBBL) were expanded, as described above, no anli-TNF producing clones were detected. However, when PBBL were sensitized, in vitro, against tetanus toxoid, boih tetanus toxoid and TNF-specific B cell clones were induced at frequencies of 1 to 5% of the clones. This data indicate that the production of anti-TNF antibodies may constitute a part of the overall B cell response to antigens.We speculate that the local production of anli-TNF Ig may protect the tumor from TNF cytotoxicity, thus allowing tumors to grow in the presence of this host reaction.

2905

Characterization of the TCR 0 chain repertoire of melanoma TILs during immunotherapy by VDJ rearrangement analysis. Puisieux I.[3], Favrot M.C.[1], Pannetier C.[2], Guillet J.G.[3], Kourilsky P.[2], Even J.[2]. [1] Centre Léon Bérard 69008 Lyon, France, [2] Pasteur U277 75015 Paris, France, [3] ICGM (Cochin) U152 75014 Paris, France.

To determine the extent of T cell receptor (TCR) usage by TILs (tumor infiltrating lymphocytes) in melanomas, the transcripts for the 24 major human Vfl families were specifically amplified by PCR after cDNA synthesis. Then the 24 VfiCll PCR products were labelled with fluorescent oligonucleotides specific of each of the 13 known Jfl segments by a cycle of elongation (run off). Sizes of the 312 run off reactions (24X13) were then measured on an acrylamide sequencing gel. The determination of the Vfl, Jfl and the size of the rearranged VDJ products allowed to classify the TCRJl repertoire according to 2500 parameters. In two melanoma patients, treated with interleukine 2, we were able to characterize T cell clones in the biopsies obtained before any treatment. First, based on the size of VDJ rearrangement, interleukine 2 enabled expansion of the same clones in regressive lesions whereas they were undetectable* in the progressive lesions. Secondly, characteristics of the clones were different in biopsies obtained from these two patients.

2906

Analysis of T-cell receptor (TCR) of tumor infiltrating lymphocytes (TILs) in neuroblastoma (NB). Valteau-Couanet, D., Carcelain, G., Leboulaire, C, Triebel, F., Hercend, T. and Hartmann, O. Institut Gustave Roussy (INSERM U333 and Department of Pediatrics), Villejuif, France

The existence of spontaneous regressions of metastasis in infants with stage IV NB and the better prognosis of tumors expressing Class I MHC molecules argue for the existence of an immune response towards NB. In order to characterize T-cells involved in tumor response, we analyzed the TCR variability in peripheral blood lymphocytes (PBL) and the primary tumor of 3 children with newly diagnosed NB. TILs were displayed by immunohistochemistry in all the 3 tumors. The expression of the TCR Va and V/? genes was studied using polymerase chain reaction with a panel of experimentally validated oligonucleotides (Voj-vi^/V^i-w^).- AH Va and \/? subfamilies were represented in PBL and tumors RNAs. In addition, some Va and V/J subfamilies appeared to be overexpressed in the tumor: Va_4, Va_{12}, Va_{13}, and \d/i in NB1; Va_{13} in NB2; *Va,* in NB3. This suggests an expansion of T-cells subpopulation following tumoral antigenic in *situ* stimulation. However, TILs did not seem to be *in situ* clonally expanded in NB1, a tumor from a 5 year-old boy, since different *la* sequences were shown by sequence analysis of Van and Va_{14} corresponding cDNAs. Regressive tumors from infants wfll be further analyzed, (supported by a LNFC grant)

2907

Isolation and functional characterization of two autologouB braaet-tumor specific CD3⁺lymphocytee clones. Nistici, P.,De Berardinis•, P., Monti, T., Del Bello, D. ,Venturo,I.,and Natali,P.G. Regina Elena Cancer Inat.,Roma;•IBPE, CNR Napoli,Italy.

To evaluate the possibility of generating breast-tumor-npecific CTL we cocultured peripheral blood lymphocytes (PBL) and autologous breast tumor (Auto-Tu) cells from a patiant with breast cancer.After cloning, two CTL clones with different phenotype, one CD3⁺aB, CD4⁺, the other one CD3⁺aB CD8⁺, selected for eytolytic activity on Auto-Tu cells and lack of lysis on the K562 cell line were functionally studied.Hot/cold experiments evidenced that the two clones preferentially recognize the Auto-Tu cellB and this cytotoxicity,while inhibited by anti LFA-1 mAb,could not be abolished by other mAbs to molecules involved in T cell function. TheBe results indicate the possibility of generating breast-tumor-specific ill CTL clones and suggest different recognition mechanisms in tumor target recognition.Supported by PF CNR ACRO and by AIRC.

2908

T cells infiltrating human renal cell carcinoma display a restricted unresponsiveness. Kudoh, S., Edinger, M., Stanley, J., Hamilton, T., Alexander, J., Tubbs, R., Klein, E., Bukowski, R., and Finke, J. The Cleveland Clinic Foundation, Cleveland, Ohio 44195

T cells (T-TIL) infiltrating renal cell carcinoma (RCC) exhibit a poor proliferative response to either IL2 or anti-CD3 antibody when compared to peripheral blood T cells. The reduced proliferative capacity of T-TIL was associated with a decrease in the induction of transferrin receptor surface expression that was measured by two color flow cytometry. Gene expression studies using reverse transcriptase-polymerase chain reaction (RT-PCR) revealed that the induction of perforin mRNA was low or not detected in T-TIL stimulated with anti-CD3. Activated peripheral blood T cells from the same patients expressed higher levels of perforin mRNA than T-TIL. In contrast, upregulation of Interleukin 2 as well as Interferon *y* mRNA was comparable in T cells from the blood and tumor. These results suggest that T-TIL have a selective loss of intracellular signalling pathways which are requisite to proliferation and perforin gene expression but not cytbkine production.

2909

Characteristics of proliferative lymph node lymphocytes selected by Radioimmunoguided Surgery in patients with colorectai cancer. Czerniecki BJ, DeGroff V, Laufman H, Martin EW Jr. Department of Surgery, The Ohio State University Hospitals and Arthur G. James Cancer Hospital and Research Institute, Columbus, Ohio 43210.

The proliferation of Radioimmunoguided Surgery™ (RIGS') selected lymph node lymphocytes (LNL) against colorectai tumor cells when activated with Interleukin 2 (IL-2, 20 u/ml) and anti-CD_3 (50 n g/ml) was assessed. These LNL contain 40% to 80% $CD_4(+)$ cells and 8% to 15% $CD_8(+)$ cells. Mixed tumor lymphocyte culture of autologous colorectai tumor cells with LNL in the presence of antibodies against Class I or Class II major histocompatibility (MHC) antigens resulted in 60% to 90% inhibition of LNL proliferation as measured by [^{3}H] thymidine incorporation. $CD_4(+)$ LNL separated by fluorescence-activated cell sorter when cultured with colorectai tumor cells with IL-2 and anti-CD_3 require the presence of adherent cells for proliferation. Both $CD_4(+)$ and $CD_5(+)$ LNL expand in short 10 day cultures. These results demonstrate that $CD_8(+)$ Class I MHC restricted lymphocytes and $CD_4(+)$ Class II MHC- restricted lymphocytes proliferate from lymph nodes identified by RIGS in patients with colorectai cancer.

C2

The Physiological and Pathological Effects of Gytokines, pages 447-452

AUTO-ANTIBODIES TO IL-lct AND TNFa IN NORMAL INDIVIDUALS AND IN INFECTIOUS AND IMMDNOINFLAMMATORY DISORDERS

K. Bendtzen, M. Syenson, A. Fomsgaard, L.K. Poulsen and M.B, Hansen

Departments of Medicine TTA and Infectious Diseases M., Rigshospitalet University Hospital, DK-2200 Copenhagen N, Denmark

INTRODUCTION

Native inhibitors of cytokines are usually low molecular weight polypeptidea. They interfere with the production or, more frequently, the function of cytokines. In the latter case, inhibitors may bind directly to the cytokine (e.g. soluble receptors) or to its receptor, or they may affect the response of target cells to the cytokine.

Native cytokine inhibitors are often nonselective in terms of function. Although this nonspecific inhibition: may be of both physiological and pathophysiological importance, specific inhibitory cytokine circuits might be even more important.

We have obtained data showing the presence of specific antibodies to IL-la and TNFa in sera from normal as well as diseased persons.

MATERIALS AND METHODS

Binding of ^{xas}1-rIL-1a to human serum immunoglobulin (Ig) was detected by column chromatography followed by co-precipitation of labelled cytokine with antibody to human Ig. Interference with ^{iSts}I-rIL-1a receptor binding was tested on murine lymphoma cells (EL 4 NOB 1) at 4°C. Ligand binding

to the cells was measured after separation from medium on oil. Binding of rTNFa to human serum Ig was detected by imraunoblotting;

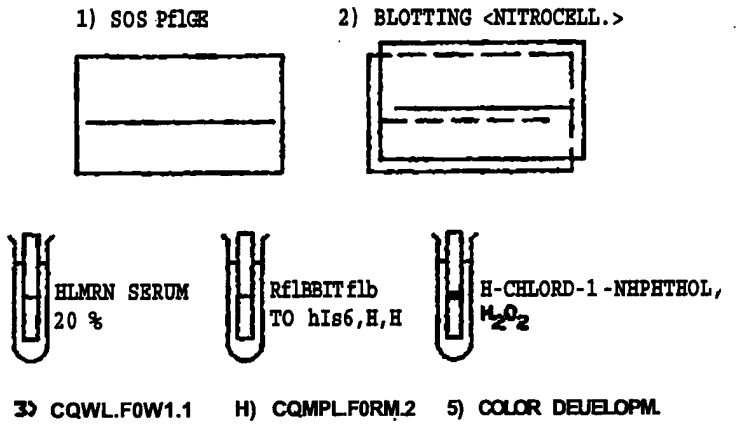


Figure 1.

The specificity of the binding was ascertained by absorption of positive sera with 10 jig/ml of rTNFa prior to assay; in all cases this prevented the antibody binding.

RESULTS

Binding of IL-la to human serum inmmnoglobulin

Sephadex G-75 column chromatography of ^{ias}i-rlL-la alone showed one peak of radioactivity at mw 17 kD. However, if ^{i3s}I-rIL«-la was incubated with human serum before chromatography, 2 peaks of activity appeared (Svenson et al., 1989). The first peak in the void volume, the other corresponding to monomer ^{ias}I-rIL-la. Sephacryl S-400 chromatography showed the high mw activity to elute in a single peak of mw 100-200 kD; 85% of this material bound to protein A. Furthermore, 94% of the ^{x*s}l activity was displaced by unlabelled rlL-la, whereas rIL-ip was ineffective. This strongly suggests that the serum factor was IgG.

Distribution of anti-IL-la IgG in normal sera

The frequency of anti-IL-la IgG was 25% in 32 normal male sera and 22% in 32 female sera when detected by co-precipitation with rabbit antibodies to human IgG. Lower binding capacities and avidities were found in the female sera (TABLE 1). Pooled anti-IL-la IgG from positive male or female sera failed to cross-bind human rIL-iP, rTNFa, rIL-6 or rIL-2.

TABLE 1. XL—k* taJLTvijjur canaci-tv of se-rivm IaG

Sex	«^{5}I-rIL-la binding*	Kd*
Hale	10 (0.7-27)	5.5 (5-7)
Female	3.3 (0.5-7.3)	11 (4-16)

~ng/ml serum; mean (range), n=5.
#pM; mean (range), n=5.

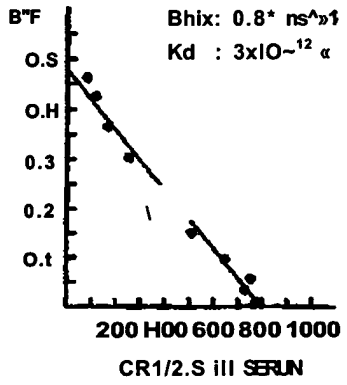


Figure 2. Schatchard analysis of the binding of ^{iaa}I-rIL-lot to normal serum IgG-. B/F is the ratio between bound and free ligand.

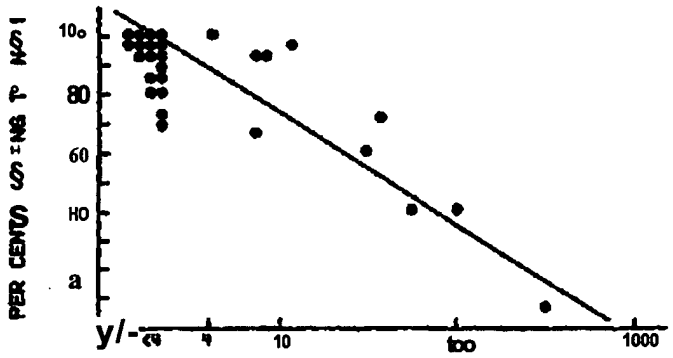


Figure 3. Receptor interference by serum antibodies to IL-lot. There was a negative correlation between antibody binding capacity and binding of ^{iaa}I-rIL-la to EL 4 cells ($r = -0.86$, $P < 0.01$).

Antibodies to TNFa in normal individuals and **patients with infectious and inflammatory diseases**

As shown in TABLE *2*, about one-third of sera from normal individuals contained detectable IgG binding to rTNFa (Forasgaard et al., 1989). The

TABLE 2.

Anti-TNFa antibodies in human sera

		% positive	
Patients	No.	Weak	Strona
Normal	50	32	8
Sepsis	38	31	32
Chronic infection	32	53	16
Inflamm. diseases	23	36	26

prevalence of these antibodies was higher in sera from patients with infectious and inflaimnatory disorders. There was no difference between the positive sera with respect to donors' age or sex. IgG antibodies (predominantly IgGi and IgGa) were found in 9 of 10 positive sera. However, in 6 of these sera IgM antibodies to rTHFa dominated. In 2 sera, IgA antibodies were also found.

TABLE 3.

Interference with ELISA for XL-1a but not TNFa by antibodv-positive sera

Serum		% of expected content					
Binding capacity[3]	cone.	Added rIL-1a 800	200 Dcr/ml=		Added rTNFa 1.000	250 Da/ml[5]	
380	50	2	1	(-)	114	100	(-)
	10	43	15	(-)	100	100	(-)
100	50	37	7	(-)	90	100	(550)
	10	102	30	(-)	100	100	(60)
8	50	102	90	(-)	nd	nd	(1500)
	10	101	100	(-)	95	120	(300)
6	50	108	102	(-)	100	100	(200)
	10	100	100	(-)	93	96	(40)
4	50	97	87	(-)	115	100	(100)
	10	105	100	(-)	88	93	(20)
4	50	101	95	(-)	95	120	(-1
	10	100	100	(-)	100	107	

[x] Estimated by second antibody precipitation of ^{125}I-rIL-1a (Svenson et al., 1989).

[3] The apparent initial serum concentrations of the cytokines, determined by ELISA, are shown in parantheses.

As shown in TABLE 3, autoantibody-positive sera interfered with ELISA for IL-1a. Sera containing immunoglobulins binding to rTNFa failed to affect ELISA for TNFa.

CONCLUSIONS

Normal sera and sera of patients with infectious or immunoinflammatory diseases contain factors, including Ig, which bind rIL-1a and rTNFa.

^{125}I-rIL-1a can be displaced from binding to the serum by rIL-1a, whereas ^{125}I-rTNFa is only partially displaced by unlabelled rTNFa (not shown). However, saturable Ig binding of rTNFa may be detected by an immunoblotting method.

Serum antibodies to IL-1a, but not to TNFa, interfere with ELISA for the respective cytokine.

IgG is the main or only rIL-1a binding Ig in sera of healthy humans. In normal sera and in sera of patients with Gram-negative bacterial sepsis or chronic inflammatory diseases IgG, IgM and IgA antibodies to rTNFa are also found.

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THE EFFECT OF PASSIVE IMMUNOTHERAPY WITH HYPERIMMUNE AND NON-HYPERIMMMUNE GAMMAGLOBULIN ON CIRCULATING ANTIGEN-SPECIFIC B CELLS IN HUMAN VOLUNTEERS JJ Drabick, K Ramsey,* JB McClain,* S Cryz,* and AS Cross** Department of Bacterial Diseases, Walter Reed Army Institute of Research, Washington, DC.

The immunomodulating abilities of gammaglobulin(IVIG) are still incompletely understood. In a recent pharmacokinetic study of a hyperimmune globulin(HIVIG) directed against antigens(Ags) of Ps. aeruginosa(PA) and Klebsiella pneumoniae (KP), we noted that post-infusion Ag-specific antibody(Ab) levels (by EIA) did not return to the pre-infusion baseline by 3 mos; some actually exhibited secondary rises in titer. This led us to postulate that specific Ab production may be actively induced by infusion of the HIVIG. To test this hypothesis, circulating Ag-specific B cells were quantified sequentially over 4 mos by the ELISPOT method. Ags tested included polysaccharides (PAT5,PAT1,KPK2), protein (PAtox a) and two negative controls (BSA, ricintox). One volunteer received HIVIG while another received IVIG (100mg/kg). The HIVIG recipient had a rise in Ag-specific IgM B cells between 10 and 20 days suggestive of a primary immune response (p=.087) compared to no response in the IVIG recipient. The response to PAtox A was significant (p=.02). We conclude that the HIVIG recipient appeared to be actively immunized by receipt of the passive HIVIG, possibly by anti-idiotype Abs present in the HIVIG which mimic the original Ags.

INCREASED GROSHONG CATHETER INFECTION RATES IN PATIENTS WITH AIDS AND HEMATOLOGIC MALIGNANCIES. DA Benator,* C Birk,* and FM Gordin* (intr. by VL Kan), Division of Infectious Diseases, Veterans Administration Medical Center, Washington, D.C.

We prospectively evaluated 188 Groshong Catheters placed in 172 patients during the period from August 1991 through September 1993 for evidence of catheter-related infection. The overall rate of infection was 0.38 per 100 patient device days (PDD). We further analyzed this data among four populations, finding a catheter infection rate of 0.72/100 PDD in patients with AIDS, 0.85/100 PDD in patients with hematologic malignancies, 0.29/100 PDD in patients with solid tumors, and 0.00 in patients without HIV or a malignancy. Coagulase-negative staphylococcus was the predominant isolate in all groups. The mean infection-free interval was 81 days to first infection, and 45 days to subsequent infections. However, as catheters that were reinfected had a shorter initial infection-free interval of 56 days, catheter replacement at the time of infection may not substantially reduce infection frequency. To our knowledge, this is the first large prospective study that looks at infection rate per patient device day in the Groshong long-term catheter. Our data confirms the findings of other indwelling catheter studies showing an increased infection rate in patients with AIDS, but also finds a similar high infection rate in patients with hematologic malignancies. Our overall infection rate was higher than in previous studies of Hickman catheters, Broviac catheters and subcutaneous ports, suggesting the need for a prospective, randomized study of infection rates in the Groshong catheter compared to other indwelling venous access devices.

EFFECT OF MONOCLONAL ANTIBODY TO HUMAN TUMOR NECROSIS FACTOR (TNF MAb) ON TNFα, IL-1ß and IL-6 LEVELS IN PATIENTS WITH SEPSIS SYNDROME. AW Chow, MG Tweeddale,* K Sleigh, K Poonia,* and H Herks*, Department of Medicine, Univ. of British Columbia and Vancouver General Hospital, Vancouver, BC.

Plasma or serum levels of TNFα, IL-1ß, and IL-6 were quantitated by ELISA in 13 patients with sepsis syndrome enroled in a double-blind, placebo-controlled, multi-center clinical trial of the efficacy and safety of TNF MAb (Bay x 1351). Patients were stratified by shock/non-shock and randomized to receive a single intravenous dose of TNF MAb (15 or 7.5 mg/kg), or placebo (0.25% human albumin). All patients received standard care in the intensive care unit. Cytokine measurements were obtained before and after TNF MAb infusion, and on days 3 and 7 after treatment.

Prior to infusion, TNFα (≥372 pg/ml) and IL-1ß (≥180 pg/ml) were detected only in 2 patients (2 of 8 with shock vs 0 of 5 non-shock; p=0.22). In contrast, IL-6 (≥316 pg/ml) was detected in all patients (3329±1906 pg/ml with shock vs 4633±821 pg/ml non-shock; p=0.06, 1-tailed t test). 2 of 3 patients who received placebo died, compared to 0 of 10 who received TNF MAb (p=0.038, Fisher's exact test). Among patients who received TNF MAb, a significant reduction in IL-6 was observed immediately following infusion (4079±1567 pg/ml PRE vs 2677±2082 pg/ml POST; p=0.05, 2-tailed paired t test), at day 3 (476±223 pg/ml, p<0.0001), and day 7 (544±462 pg/ml, p<0.0005). Insufficient patients were enroled for this comparison in the placebo group. No differences in TNFα, IL-1ß or IL-6 levels were observed between patients who received 15 mg/kg (n=6) vs 7.5 mg/kg (n=4) TNF MAb.

Thus, in this limited sample, there is an inverse association of plasma/serum IL-6 levels and shock in patients with sepsis syndrome, suggesting a possible protective effect of IL-6 for septic shock. Furthermore, IL-6 appears more sensitive than either TNFα or IL-1ß for monitoring the severity and outcome of sepsis syndrome patients during experimental therapy.

MOLECULAR DETECTION OF LATENT MURINE CYTOMEGALOVIRUS (MCMV) DNA IN HEPATIC SINUSOIDAL ENDOTHELIAL CELLS. T. Collins,* M. Quirk,* K. Cleary,* H. Sharp, and M.C. Jordan, Departments of Medicine & Pediatrics, University of Minnesota, Minneapolis, MN.

Transmission of latent human cytomegalovirus (CMV) from donor to recipient by liver transplantation occurs at a frequency greater than with any other organ. In the murine model of CMV, the non-replicating latent infection has been characterized most extensively in the spleen where it is maintained in stromal cells within the red pulp. Using a nested polymerase chain reaction (PCR) to amplify a 200 bp region of DNA in exon 4 of the immediate early gene (IE-1), we have previously detected MCMV DNA in the liver of all latently infected mice. Experiments were undertaken to examine our hypothesis that latent MCMV would reside primarily in the stromal regions of the liver as in the spleen. Purified liver cell populations (hepatocytes, endothelial cells, Kupffer cells, bile duct/Ito cells) were obtained by elutriation techniques from Balb/c mice with latent MCMV infection, and DNA and RNA were extracted. Latent MCMV DNA was detected using the nested PCR in 10/12 samples of sinusoidal endothelial cells, 0/12 Kupffer cells, 2/6 hepatocytes, and 2/6 Ito/bile duct cells (endothelial vs. Kupffer cells significant $p < 0.0001$). To determine whether the lower frequency of detection of viral DNA in the hepatocytes and Ito/bile duct cells was due to contamination with endothelial cells, combined PCR and *in situ* DNA hybridization was performed. Individual cell suspensions were fixed in paraformaldehyde and subjected to enzymatic amplification of latent MCMV DNA. Afterwards, cell suspensions were cytocentrifuged, and the 200 bp PCR product was sought by *in situ* hybridization with a ^{35}S labeled, single-stranded riboprobe. In three consecutive experiments the 200 bp PCR product was detected exclusively in sinusoidal endothelial cells. To determine whether the IE-1 gene of MCMV was expressed during latency, total RNA recovered from each liver cell population was incubated with Moloney leukemia virus reverse transcriptase and an antisense IE-1 primer to generate MCMV IE-1 cDNA. The cDNA subsequently was amplified using a nested PCR. No immediate early transcripts were detected in any liver cell population. These results indicate that latent MCMV resides within the hepatic sinusoidal endothelial cells and that the IE-1 gene is either not expressed during latency or is expressed at a level below the sensitivity of the assay used.

DIMETHYLTHIOUREA PROTECTS RATS AGAINST GRAM-NEGATIVE SEPSIS BY DECREASING TUMOR NECROSIS FACTOR ACTIVITY. RC Sprong,* JM Aarsman,* AML Janssen,* JH van Kats,* and BS van Asbeck,** Departments of Internal Medicine and Medical Microbiology, University Hospital, Utrecht, The Netherlands.

The thiol containing compound dimethylthiourea (DMTU) is known to be protective in various models of endotoxin (LPS)-mediated tissue damage. DMTU might exert its effect by reducing oxidative stress. Alternatively, DMTU could inhibit TNF activity, as is recently shown for the oxygen radical scavenger N-acetylcysteine. In this study, we evaluated the effect of DMTU on survival, oxidative stress and TNF activity in two rat models of gramnegative sepsis. When rats (n=26) were intraperitoneally injected with 500 mg DMTU/kg together with 5 mg LPS/kg, 81% was still alive after 24h versus 53% in control rats (n=39; $p < 0.05$). Treatment with DMTU also diminished the deadly effect of i.p. injected live 3 x 10^{10} *S.typh.*/kg (40% survival vs. 0% in saline treated rats; $p < 0.05$). LPS injection resulted in oxidative stress, as indicated by an elevated concentration of H_2O_2 in deproteinized blood (374 ± 119 μM at t=1.5h vs. 226 ± 93 μM at t=0h; $p < 0.01$). Similarly, we also observed an elevated peroxide tonus in animals injected with *S. typh.* (525 ± 74 μM; $P < 0.05$). Although DMTU improves survival in both models, peroxide concentrations were not affected by DMTU, which is consistent with our in vitro observation that DMTU is a weak H_2O_2 scavenger but powerfully inactivates hydroxyl radical (·OH). Serum TNF activity, however, was substantially decreased in DMTU treated rats ($p < 0.05$). This was in agreement with an inhibitory effect of DMTU on LPS-induced binding of a nuclear factor-kappa B (NF-*k*B) like transcription factor to the NF-*k*B consensus locus of the rat TNF gene. This study indicates that oxidative stress is induced in gramnegative sepsis, and that the ·OH scavenger DMTU protects by decreasing TNF activity.

GENETIC DETERMINANTS OF PERSISTENT REPLICATION OF HUMAN CYTOMEGALOVIRUS IN HUMAN FETAL TISSUE. JM Brown, ES Mocarski (intr. by by AR Hoffman), Departments of Microbiology/Immunology and Medicine, Stanford University, Stanford, CA.

Human cytomegalovirus (HCMV) is major cause of morbidity and mortality in immunosuppressed patients. The escalating incidence of acute infection in young children and their parents may lead to an increased incidence of intrauterine transmission with its debilitating sequelae. Epidemiological studies suggest that different strains of HMCV differ in their virulence potential. However, the high degree of species specificity exhibited by HCMV has hampered its direct study. We report HCMV infection of human fetal tissues that have been implanted in the severe combined immunodeficient mouse, with the resulting chimera known as the SCID-hu mouse. Toledo, a clinical strain known to cause disease, was able to persistently replicate for months following inoculation of human fetal thymic tissue, in contrast to a highly laboratory-passaged vaccine candidate strain AD169, that failed to grow. Virus was localized to the thymic epithelial cells (TECs) of the medulla; therefore, TECs were immortalized using a recombinant amphotropic retrovirus containing the E6/E7 genes of human papillomavirus, type 16. Immunofluorescent antibody identification of early and late gene products and assay of HCMV replication in immortalized TECs paralleled the results in the intact tissue. Interstrain genomic differences have been characterized using restriction fragment length polymorphisms and hybridization analysis. AD169 has a sizable deletion when compared to Toledo and other strains able to persistently replicate. In conclusion, we report (1) the direct study of HCMV infection in human tissue and interstrain differences in persistent replication; (2) the development of immortalized clonal lines of TECs which demonstrate a heterogeneity in their permissiveness for HCMV infection, (3) in clonal TEC populations that reflect interstrain differences in replication, viral replication appears blocked after viral entry and (4) the identification of interstrain genomic differences that correlate with the ability to persistently replicate.

C6

A Specific Molar Ratio of Stabilizer to Protein is Required for Storage Stability of a Lyophilized Monoclonal Antibody

JEFFREY L. CLELAND,[1] XANTHE LAM,[1] BRENT KENDRICK,[2] JANET YANG,[3] TZUNG-HORNG YANG,[4] DAVID OVERCASHIER,[1] DENNIS BROOKS,[1] CHUNG HSU,[1] JOHN F. CARPENTER[4]

[1]Pharmaceutical R & D, Genentech, Inc., 1 DNA Way, South San Francisco, California 94070

[2]Pharmaceutics Department, Amgen, Inc., Thousand Oaks, California

[3]Quality Control, Genentech, Inc., South San Francisco, California

[4]Department of Pharmaceutical Sciences, University of Colorado Health Sciences Center, Denver, Colorado

Received 22 June 2000; revised 25 September 2000; accepted 26 September 2000

ABSTRACT: The selection of the appropriate excipient and the amount of excipient required to achieve a 2-year shelf-life is often done by using iso-osmotic concentrations of excipients such as sugars (e.g., 275 mM sucrose or trehalose) and salts. Excipients used for freeze-dried protein formulations are selected for their ability to prevent protein denaturation during the freeze–drying process as well as during storage. Using a model recombinant humanized monoclonal antibody (rhuMAb HER2), we assessed the impact of lyoprotectants, sucrose, and trehalose, alone or in combination with mannitol, on the storage stability at 40°C. Molar ratios of sugar to protein were used, and the stability of the resulting lyophilized formulations was determined by measuring aggregation, deamidation, and oxidation of the reconstituted protein and by infrared (IR) spectroscopy (secondary structure) of the dried protein. A 360:1 molar ratio of lyoprotectant to protein was required for storage stability of the protein, and the sugar concentration was 3–4-fold below the iso-osmotic concentration typically used in formulations. Formulations with combinations of sucrose (20 mM) or trehalose (20 mM) and mannitol (40 mM) had comparable stability to those with sucrose or trehalose alone at 60 mM concentration. A formulation with 60 mM mannitol alone provided slightly less protection during storage than 60 mM sucrose or trehalose. The disaccharide/mannitol formulations also inhibited deamidation during storage to a greater extent than the lyoprotectant formulations alone. The reduction in aggregation and deamidation during storage correlated directly with inhibition of unfolding during lyophilization, as assessed by IR spectroscopy. Thus, it appears that the protein must be retained in its native-like state during freeze–drying to assure storage stability in the dried solid. Long-term studies (23–54 months) performed at 40°C revealed that the appropriate molar ratio of sugar to protein stabilized against aggregation and deamidation for up to 33 months. Therefore, long-term storage at room temperature or above may be achieved by proper selection of the molar ratio and sugar mixture. Overall, a specific sugar/protein molar ratio was sufficient to provide storage stability of rhuMAb HER2.

J Pharm Sci 90:310–321, 2001
Keywords: protein stability; lyophilization; monoclonal antibody; aggregation; sugars

Correspondence to: J.L. Cleland (Telephone: 650-225-3921; Fax: 650-225-3979; E-mail: cleland@gene.com)

Journal of Pharmaceutical Sciences, Vol. 90, 310–321 (2001)

INTRODUCTION

The successful development of therapeutic proteins depends on the ability to deliver them in

their intact native state. Proteins are often formulated in aqueous solution to allow ease of use, but several degradation processes may be facilitated by the aqueous environment.[1] To obtain the desired stability during storage for up to 2 years, proteins are often dried to reduce the rate of chemical and physical degradation.[2,3]

There have been numerous reports of the ability of sugars, such as sucrose and trehalose, to stabilize proteins during lyophilization and storage in the dried solid.[4–8] Recent studies have also shown the utility of these sugars in producing freeze-dried,[9–11] spray freeze-dried, or spray-dried antibodies.[12,13] The mechanism of stabilization by these sugars during drying has been proposed to occur by producing a glassy matrix to restrict mobility and/or acting as a water substitute (reviewed by Carpenter et al.[14–16]). Several recent cases have shown that a glassy matrix alone is not sufficient to inhibit protein unfolding during dehydration and that hydrogen bonding of the sugar to the dried protein (water substitution) is a requisite for inhibition of lyophilization-induced protein unfolding.[5–7,17,18]

The removal of water in the absence of a stabilizing agent, such as a sugar, often results in a non-native protein structure in the dry state and may lead to aggregation during reconstitution.[4,17,19] Furthermore, it has also been shown with a few proteins that retention of the native protein structure in the initial lyophilized cake is important for optimal long-term storage stability in the dried solid (reviewed by Allison et al.[8] and Carpenter et al.[15,16]). In addition, it has been found that sugars provide a glassy matrix that is required to reduce the rate of degradative reactions during storage in the dried solid.[6,7,9,16,17]

Many stability studies are performed using isoosmotic concentrations of sugars (e.g., 275 mM sugar) and do not determine the minimum concentration required for optimal long-term stability. In the current investigation, to determine the optimum sugar content for protein stability, we studied the effect of a wide range of sugar-to-protein molar ratios, as well as different sugars, on the retention of native protein structure in the dried solid and prevention of degradation during storage. To assess the relationship between protein structure in the dried state and long-term storage stability, protein secondary structure was analyzed by infrared (IR) spectroscopy and the levels of aggregated and deamidated protein were determined in reconstituted samples.

The model protein selected for these studies was the recombinant humanized monoclonal antibody against the human epidermal growth factor receptor-2 (rhuMAb HER2), which has recently been approved by the U.S. Food and Drug Administration for treatment of metastatic breast cancer. This antibody has a molecular weight of ~150 kDa, and is composed of a human Fc domain of the subtype IgG_1 with humanized framework and complementary determining regions (CDRs) designed to provide comparable binding affinity to the murine form (see Carter et al.[20] for details on the humanization process). Previous studies by our group[21] and others[22] have shown that this antibody is susceptible to oxidation at Met-255 and Met-431 in the Fc domain. Deamidation and formation of isoaspartate and succinimide may also occur at Asn30 in the light chain and Asn102 in the heavy chain.[23] These reactions may be slowed or eliminated by the removal of water to produce a freeze-dried product. However, the removal of water may foster denaturation leading to another degradation route, aggregation. A successful formulation of rhuMAb HER2 must therefore provide protection against aggregation during lyophilization and storage, as well inhibition of chemical degradation. In the studies described herein, we have used storage at elevated temperature (40°C) to determine the relative stability of several formulations. We have also investigated conditions that are hypotonic (<275 mM) and may be used for formulations that require dilution into intravenous (iv) saline bags or reconstitution with volumes that are less than the fill volume yielding a more concentrated solution.

MATERIALS AND METHODS

Materials

Recombinant humanized monoclonal antibody against the human epidermal growth factor receptor 2 (rhuMAb HER2) was expressed in Chinese hamster ovary (CHO) cells and purified at Genentech, Inc. The purification was performed using Protein A affinity chromatography followed by cation exchange to remove aggregates and charged forms of the antibody. The final purified antibody was provided in 5 mM sodium histidine, pH 6 by Genentech's Product Recovery Operations.

Methods

Size Exclusion Chromatography

Distribution of intact protein, soluble aggregate and/or fragments of rhuMAb HER2 was determined using size exclusion chromatography (SEC). A high-performance liquid chromatography (HPLC) system (Hewlett Packard 1090L) was used with a TSK 3000 SWXL (Toso Haas Corporation) column at a flow rate of 1 mL/min using pH 7.2 phosphate-buffered saline (PBS) as the mobile phase. A 25-μg sample of protein was injected onto the column and detected by absorbance at 280 nm. Standards of known protein concentration were run as controls for each set of samples. Samples were run twice, and the average value was used to determine the fraction of aggregated protein in the sample [coefficient of variation (CV) ± 5%]. The fractions of aggregate, intact protein, and protein fragments were determined by assessing the relative peak areas of each species. For SEC, ion-exchange, and hydrophobic interaction methods, three vials were assayed for each time point and the results were reported as a mean and standard deviation (SD). All chromatographic methods yielded complete protein recovery (no protein loss) when compared with appropriate controls.

Cation-Exchange Chromatography

The amounts of deamidated cyclic imide and intact rhuMAb HER2 were quantitatively determined by cation-exchange chromatography (IEX). A wide-pore carboxy sulfon column was heated to 40°C, and chromatography was performed at a flow rate of 1 mL/min. Separation of various charged species was achieved through a NaCl gradient in 20 mM Na phosphate buffer (pH 6.9). Samples were diluted to 1 mg/mL protein, and then 75 μg of protein was injected onto the column and detected by absorbance at 214 nm. All samples were analyzed in duplicate along with appropriate standards (CV ± 5%).

Hydrophobic Interaction Chromatography

The detection of rhuMAb HER2 oxidized species was achieved by digestion with carboxypeptidase B and papain. The resulting Fab and Fc fragments were analyzed by hydrophobic interaction chromatography (HIC) using a TSK Butyl-NPR column (4.6 × 35 mm, Tosohaas) and a gradient from 0 to 2 M ammonium sulfate as previously described.[21]

Residual Moisture of Lyophilized Cakes

Residual moisture of lyophilized rhuMAb HER2 was determined by the Karl Fisher (Brinkmann, model 658) titration method. Measurements were made by an electrochemical technique that utilizes the conductive state of a hydranal composite/methanol solution. Samples for residual moisture analysis were prepared by dispersing the lyophilized cake to a powder in the vials. The percentage by weight of residual water moisture present was determined for sample weights ranging from 40 to 70 mg. A mean titer standard (CV ± 5%) derived from ten 5-μL water samples was used to calculate the percentage by weight of residual water present in the samples. Two or three moisture measurements were conducted per vial depending on the availability of material. Samples for excipient screening all had residual moisture contents of ≤ 2% w/w (g water/100 g solid).

pH of Frozen Protein Solution

The pH of rhuMAb HER2 formulated at 25 mg/mL in 60 mM trehalose, 5 mM histidine, pH 6.3, and 0.01% polysorbate 20 was determined at −20°C (frozen) using a combined pH electrode (Mettler Toledo, Wilmington, MA) as described previously.[24] The probe was calibrated at 25, 10, and 0°C with buffer standards at pH 4, 7, and 10 (Baxter Diagnostics), and the SD of the measurements were ± 0.10 pH units.

Infrared Spectroscopy

The IR spectra of native aqueous protein and protein in lyophilized formulations were acquired and processed as previously described using a Bomem Prota Spectrometer and software.[6–8,18]

Differential Scanning Calorimetry

Differential scanning calorimetric (DSC) analysis of lyophilized formulations was conducted as previously described[6–8,18,25] with a Perkin Elmer DSC-7, and the percentages of crystalline mannitol were determined as detailed previously.[25]

Experimental Conditions

The protein was formulated at 25 mg/mL in either 5 mM succinate, pH 5.0 or 5 mM histidine, pH 6

with 0–250 mM trehalose and 0.01–0.2% w/v polysorbate 20. Additional studies were performed within this range of polysorbate 20 concentrations, and no effect was observed on the stability of rhuMAb HER2 following storage at 40°C for 6 months in formulations containing 60 mM trehalose and 5 mM histidine, pH 6 (data not shown). Additional solutions were prepared at the same protein concentration in 5 mM histidine, pH 6 with 0–250 mM sucrose and 0.1% polysorbate 20. To determine the effects of mannitol on stability of rhuMAb HER2, the protein was also formulated at 25 mg/mL in 5 mM histidine, pH 6 and 0.1% polysorbate 20 with 60 mM mannitol, and with combinations of mannitol and sucrose (55 mM mannitol/5 mM sucrose; 50 mM mannitol/10 mM sucrose; 40 mM mannitol/20 mM sucrose). The effect of protein concentration on stability was determined by formulation of rhuMAb HER2 at 25 or 50 mg/mL in either 60 mM trehalose, 20 mM trehalose/40 mM mannitol, or 20 mM sucrose/40 mM mannitol with 5 mM histidine, pH 6 and 0.1% polysorbate 20. Long-term stability of the lyophilized protein at 40°C was also determined for formulations containing trehalose alone or the mannitol/lyoprotectant combinations at 5 mg/mL rhuMAb HER2 in 5 mM histidine, pH 6 and 0.01% polysorbate 20. Each study was performed on a single lot of each formulation.

Lyophilization

For each formulation, 18 mL of solution was filled into depyrogenated, sterile 50-cc vials and lyophilized in a GT20 freeze-dryer. Primary drying was conducted at shelf temperatures of −10–5°C for 40–68 h (greater time for lower temperatures) and secondary drying was performed for 10–12 h at a shelf temperature of 20°C. For residual moisture studies, vials were removed at different times during secondary drying using a special access port to allow maintenance of vacuum. The chamber pressure was maintained constant at 150 mTorr throughout primary and secondary drying. Vials were capped under partial vacuum. Reconstitution was performed using 20 mL of Sterile Bacteriostatic Water for Injection (SBWFI) containing 1.1% benzyl alcohol. SBWFI was chosen to allow multiple withdrawals of the protein from the vial for repeated administration from a single vial (multidose vial). A formulation consisting of 25 mg/mL rhuMAb HER2, 60 mM trehalose, 5 mM histidine, pH 6, and 0.01% polysorbate 20 was placed at 40°C for 6 months, and vials were reconstituted with either sterile water for injection (SWFI) or SBWFI. This study demonstrated that reconstitution with SBWFI or SWFI yielded equivalent stability.

Stability Assessment

Control samples of each formulation before lyophilization were retained (stored frozen) and analyzed at the same time as lyophilized samples. Each formulation was reconstituted and tested immediately after lyophilization. Each formulation was stored at 40°C for 1 and 3 months in the lyophilized state and then reconstituted and analyzed. Long-term stability (23–55 months) was determined for selected formulations.

RESULTS AND DISCUSSION

A variety of different sugars and molar ratios of sugar to protein were used to produce lyophilized rhuMAb HER2 formulations. As noted in Table 1, after lyophilization and immediate reconstitution (no storage), even in the absence of sugars, there was only a slight increase in aggregate formation. However, on storage of these lyophilized formulations at 40°C, the extent of aggregation increased dramatically (Figures 1 and 2). Without sugars, the extent of rhuMAb HER2 aggregation increased from ~1% to > 10% after 3 months at 40°C. In the absence of sugar, a greater extent of aggregation was observed in the histidine formulation than in the succinate formulation after 3 months at 40°C (Figure 1). Previous studies had indicated the potential for histidine to provide stabilizing effects during lyophilization and storage,[26] but this stabilization was not observed with rhuMAb HER2.

The addition of sucrose or trehalose in the formulations reduced the amount of aggregation noted after storage and reconstitution (Figures 1 and 2). There was no apparent difference in the stabilizing affect of trehalose or sucrose (Figure 2b). The amount of aggregated protein decreased with increasing sugar concentration up to 100 mM, and only minor reductions in the amount of aggregate over 3 months of storage at 40°C were observed for formulations containing > 60 mM sugar. At 60 mM sugar, the molar ratio of sugar to protein was 360. Similar stoichiometric amounts of carbohydrates were required to stabilize another recombinant antibody during spray drying and subsequent storage.[12]

Table 1. Effect of Lyophilization on the Aggregation of rhuMAb HER2[a]

Formulation	% Aggregate	
	Before Lyophilization	After Lyophilization
5 mM Succinate, pH 5	0.2 ± 0.1	1.4 ± 0.2
+ 60 mM trehalose	0.0 ± 0.0	0.0 ± 0.0
+ 100 mM trehalose	0.0 ± 0.0	0.0 ± 0.0
+ 150 mM trehalose	0.0 ± 0.0	0.0 ± 0.0
+ 200 mM trehalose	0.0 ± 0.0	0.0 ± 0.0
+ 250 mM trehalose	0.7 ± 0.2	0.9 ± 0.3
5 mM Histidine, pH 6	0.4 ± 0.2	1.1 ± 0.3
+ 30 mM trehalose	0.3 ± 0.1	0.5 ± 0.2
+ 60 mM trehalose	0.0 ± 0.0	0.0 ± 0.0
+ 100 mM trehalose	0.0 ± 0.0	0.0 ± 0.0
+ 150 mM trehalose	0.0 ± 0.0	0.0 ± 0.0
+ 200 mM trehalose	0.0 ± 0.0	0.0 ± 0.0
+ 250 mM trehalose	0.0 ± 0.0	0.0 ± 0.0
5 mM Histidine, pH 6	—	—
+ 60 mM sucrose	0.5 ± 0.1	1.0 ± 0.1
+ 100 mM sucrose	0.4 ± 0.2	0.3 ± 0.1
+ 150 mM sucrose	0.2 ± 0.1	0.6 ± 0.2
+ 200 mM sucrose	0.7 ± 0.1	0.4 ± 0.2
+ 250 mM sucrose	0.6 ± 0.2	0.5 ± 0.2
5 mM Histidine, pH 6		
+ 60 mM mannitol	0.5 ± 0.2	0.4 ± 0.1
+ 55 mM mannitol/5 mM sucrose	0.4 ± 0.3	0.9 ± 0.2
+ 50 mM mannitol/10 mM sucrose	0.0 ± 0.0	0.5 ± 0.1
+ 40 mM mannitol/20 mM sucrose	0.0 ± 0.0	0.0 ± 0.0

[a] All formulations consisted of 25 mg/mL protein and were lyophilized as described in the MATERIALS AND METHODS section.

To determine if this stabilizing phenomenon was specific to sucrose and trehalose, mannitol was used in the formulation buffer at 60 mM (the apparent minimum sugar concentration required for stability). Although storage of the mannitol formulation at 40°C for 3 months resulted in less aggregation than the formulations without sugar, the mannitol formulation provided slightly less protection against aggregation than the same concentration of sucrose or trehalose (Figures 1–3). Previous studies have indicated that mannitol may crystallize during lyophilization, forming a separate phase from the protein.[6,27–19] If this phase separation had occurred, lyophilization-induced unfolding may not be inhibited (vide infra) and the amount of aggregated protein in the mannitol formulations should be comparable to or greater than that in the formulations without sugar. However, this low concentration of mannitol may remain amorphous and in the same phase as the protein during drying, providing protection to the protein. In support of this suggestion, DSC analysis (cf., Carpenter et al.[25]) determined that mannitol in the lyophilized 60 mM mannitol rhuMAb HER2 formulation was only 7% crystalline (data not shown).

Furthermore, the presence of sugars that remain amorphous during lyophilization (e.g., sucrose) in mannitol formulations can further inhibit mannitol crystallization.[30] Several studies have also shown that combinations of mannitol and an amorphous excipient improve protein stability after lyophilization and storage relative to that noted with mannitol alone.[31–33] To test for this effect, sucrose was added to the formulation to yield a range of sucrose and mannitol/protein molar ratios. The use of 20 mM sucrose (sucrose/protein molar ratio of 120:1), which was not optimum for stability when used alone, and 40 mM mannitol was sufficient to provide stabilization against aggregation after storage and reconstitution, yielding comparable stability to 60 mM

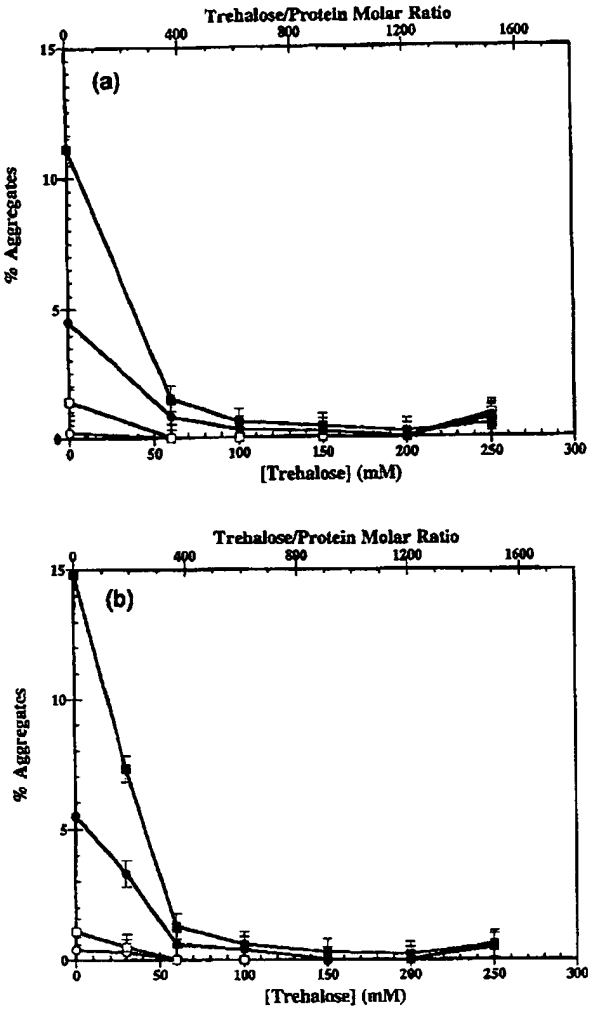

Figure 1. The effect of trehalose concentration on the fraction of aggregated protein after reconstitution of lyophilized formulations was measured by SEC. rhuMAb HER2 was formulated at 25 mg/mL in either 5 mM succinate, pH 5 (A) or 5 mM histidine, pH 6 (B). Samples were analyzed prior to lyophilization (open circles), immediately after lyophilization (open squares), or after storage of the lyophilized protein for 1 (closed circles) or 3 (closed squares) months at 40°C.

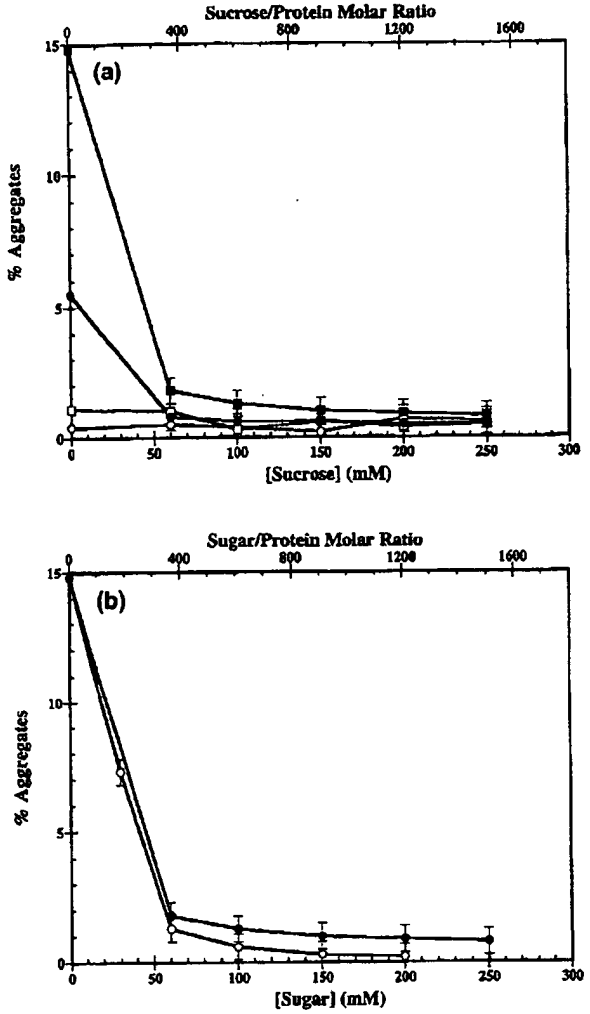

Figure 2. The effect of sucrose concentration on the fraction of aggregated protein after reconstitution of lyophilized formulations was measured by SEC. rhuMAb HER2 was formulated at 25 mg/mL in 5 mM histidine, pH 6. (A) Samples were analyzed prior to lyophilization (open circles), immediately after lyophilization (open squares), or after storage of the lyophilized protein for 1 (closed circles) or 3 (closed squares) months at 40°C. (B) Sucrose (closed circles) and trehalose (open circles, 5 mM histidine, pH 6) formulations provided comparable protection against aggregation upon stored at 40°C for 3 months.

sucrose alone (Figures 2 and 3). Stabilization of lyophilized proteins formulated with mannitol in combination with glycine,[31,34] sucrose, or trehalose[33] have been previously reported and indicate that mannitol provides some protection if it remains in the amorphous form.[32] These studies suggest that mannitol may either facilitate sucrose stabilization of the protein or directly stabilize the protein when maintained in the protein phase during drying (amorphous form). With the lyophilized 20:40 mM sucrose/mannitol rhuMAb HER2 formulation, DSC analysis indicated that only 4% of the mannitol was crystalline.

The effects observed in these experiments may have resulted from the overall concentration of the sugars used in these studies and not the specific molar ratio of sugar to protein. Different rhuMAb HER2 concentrations were therefore

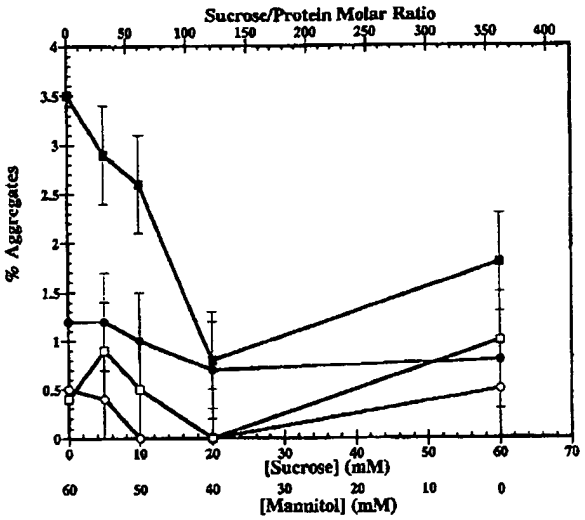

Figure 3. The stability of rhuMAb HER2 formulated in mannitol or mannitol/sucrose formulations was determined by measuring aggregation by SEC after reconstitution of lyophilized formulations. rhuMAb HER2 was formulated at 25 mg/mL in 5 mM histidine, pH 6. Samples were analyzed prior to lyophilization (open circles), immediately after lyophilization (open squares), or after storage of the lyophilized protein for 1 (closed circles) or 3 (closed squares) months at 40°C.

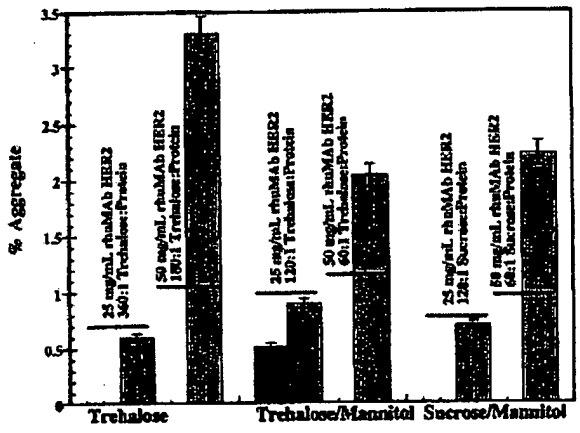

Figure 4. Effect of protein concentration on aggregation of rhuMAb HER2 in lyophilized formulations was assessed after reconstitution either immediately after lyophilization (solid bars) or after storage of the lyophilized protein for 1 month at 40°C.

studied to assess the mechanism of sugar stabilization. Maintaining the trehalose concentration at 60 mM and increasing the protein concentration from 25 to 50 mg/mL resulted in a significant increase in aggregation on storage for only 1 month at 40°C (Figure 4). As a result of this increase in protein concentration, the molar ratio of sugar to protein decreased from 360 to 180. The extent of aggregation at a molar ratio of sugar to protein of 180 was comparable to that observed for 30 mM trehalose and 25 mg/mL rhuMAb HER2 (molar ratio of 180, Figure 1), suggesting that the molar ratio is critical for stabilization of the protein during storage. The mixed sugar systems of 20:40 mM trehalose/mannitol and 20:40 mM sucrose/mannitol yielded similar results where a reduction from a molar ratio of trehalose or sucrose to protein from 120 to 60 yielded a marked increase in aggregation after 1 month of storage at 40°C. Overall, these data revealed that the molar ratio of sugar to protein appears to be a critical variable for protein stabilization during lyophilization and storage.

Long-term protein storage at high temperatures (40°C) was performed on selected samples to further test the impact of the sugar/protein molar ratio on protein stability (Table 2). At very high sugar/protein molar ratios (600–1800:1), only a small increase in aggregation was observed after 33 months at 40°C. At the intermediate molar ratio of 360:1, there was an increase to ~10% aggregation after > 50 months. The rate of aggregation at 40°C for these formulations was ~0.2% per month at 40°C, assuming zero-order kinetics. For a requirement of > 95% monomer at the end of shelf-life, this formulation may be stored at 40°C for 18 months. Decreasing the molar ratio to 180:1 caused a dramatic increase in aggregation (29.9%) after 44 months at 40°C. At the appropriate molar ratio, trehalose and sucrose or their combinations with mannitol may provide long-term, room-temperature stability, and perhaps even formulations that do not require refrigeration even at relatively high ambient temperatures.

Although the initial protein formulations used in the studies just described contained ≤ 2% residual moisture, the impact of 1–8.4% residual moisture was studied for the formulation containing 60 mM trehalose, 5 mM histidine, pH 6, and 0.01% polysorbate 20 to assess the impact of moisture on protein stability. There was no effect of residual moisture over this range on the rate of aggregation during storage at 2–8 or 40°C for 12 months. These data suggest that moisture level differences (1–2% moisture in all formulations) between the formulations did not affect aggregation during storage.

Table 2. Effect of Excipients on the Long-Term Stability of rhuMAb HER2 at 40°C[a]

[Protein] (mg/mL)	Excipient	[Excipient] mM	Molar ratio Excipient: Protein	Storage Time (mo.)	SEC Analysis		IEX Analysis
					% Aggregate	% Monomer	% Main Peak
5	Sucrose	20	600	33	1.1	98.9	80.8
	Mannitol	40	1200				
5	Trehalose	20	600	33	1.7	98.9	81.7
	Mannitol	40	1200				
5	Trehalose	60	1800	33	0.8	99.2	80.1
50	Trehalose	100	300	23	9.7	90.3	76.5
50	Trehalose	60	180	44	29.9	70.1	74.0
25	Trehalose	60	360	51	8.5	91.6	79.8
25	Trehalose[b]	60	360	54	11.0	89.0	76.6
25	Trehalose[c]	60	360	—	0.1	99.9	80.5

[a] All formulations contained 5 mM histidine, pH 6.0, unless otherwise noted. Because of sample limitations, single vials were assayed in duplicate and the average values are reported (CV ± 4%).
[b] Formulation was prepared in 5 mM succinate, pH 5.0.
[c] Reference standard stored at 2–8°C.

Secondary Structure of Lyophilized Protein

To determine the relationship between protein structure in the dried solid and protein stability during storage, the effects of excipients on the secondary structure of lyophilized rhuMAb HER2 (25-mg/mL formulations) were investigated with second-derivative IR spectroscopic analysis. As expected for a monoclonal antibody, the spectra for the aqueous native protein, in the conformationally sensitive amide I region, was dominated by bands at 1640 and 1690 cm^{-1}, which are due to the β-sheet structure (Figure 5). Bands were also observed at 1665 and 1680 cm^{-1}, which are due to the turn structure. When the protein was lyophilized in the absence of sugar, the resulting IR spectra in the dried solid indicated a substantial perturbation of secondary structure, which can be seen as a large decrease in the intensity, a broadening of the band at 1640 cm^{-1}, and a shift in the high frequency β-sheet band to 1695 cm^{-1} and an increase its area (Figure 5). In the presence of 60 mM sucrose, these transitions were partially inhibited, as indicated by the increase in intensity at 1640 cm^{-1}, a shift in the high-frequency β-sheet band to 1693 cm^{-1}, and a decrease in area of this band. In the presence of 60 mM mannitol, the degree of structural protection was slightly less than that noted for 60 mM sucrose (Figure 5). The sample with 20 mM sucrose and 40 mM mannitol had a spectrum that was also very similar to that for the sample with 60 mM sucrose (data not shown). With sucrose alone, the maximal degree of structural protection was obtained with a 100 mM concentration, based on absorbance at 1640 cm^{-1} (Figure 5). Increasing sucrose concentrations to 200 mM did not result in further increases in structural protection (data not shown). Similar results were noted with trehalose, where the maximal degree of retention of native structure also occurred at 60 mM initial sugar concentration (data not shown).

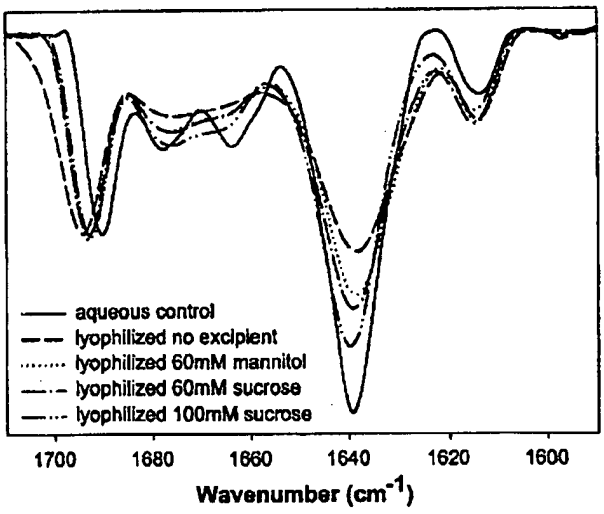

Figure 5. Second-derivative IR spectra were obtained for rhuMAb HER2 formulations: liquid control (5 mM histidine, pH 6, 60 mM trehalose), and lyophilized solids (5 mM histidine, pH 6 without sugar or with 60 mM sucrose, 100 mM sucrose, or 60 mM mannitol).

The spectra of all the formulations taken after 3 months of storage at 40°C, had only slightly reduced absorbance at 1640 cm^{-1} relative to that noted in the respective formulations immediately after lyophilization (data not shown). Also, in the spectra for the stored samples, as was the case for the spectra of samples immediately after lyophilization, bands indicative of intermolecular β-sheet structure (e.g., 1622 cm^{-1}) were not present. Similar results were found with the long-term stored samples that were analyzed by IR spectroscopy (data not shown), which included all formulations shown in Table 2, except the trehalose sample with an excipient/protein molar ratio of 180:1. Unfortunately, the single vial of this sample, which had the highest level of aggregation noted in the current study, was reconstituted before investigation of the dried solid by IR spectroscopy. Taken together, the IR spectroscopic and aggregation results document that either aggregation was not occurring in the dried solid and/or that the level of intermolecular β-sheet present in samples containing up to 15% aggregated rhuMAb HER2 is not sufficient to be detected by solid-state IR spectroscopy.

The capacities of the sugars to inhibit protein unfolding during lyophilization and aggregation during storage/rehydration were almost maximal at 60 mM initial sugar concentration; only a slight improvement is noted by increasing concentration to 100 mM sucrose concentration (Figures 2b and 5). Thus, it appears that optimal storage stability noted with a minimal excipient/protein molar ratio of 360 is due in large part to the retention of native protein structure in the initial lyophilized solid.

Another physical factor that has been found to be important in determining the stability of lyophilized protein formulations during long-term storage is the glass transition temperature (T_g) of the amorphous phase containing the protein.[6–9,14–17] Storage of samples above T_g can lead to rapid degradation.[6–9,14–17] The T_g values for a limited number of the formulations, for which stability results are presented in Figures 1 and 2, were determined by DSC. In all cases, the measured T_g values were >60°C, which far exceeds the storage temperature of 40°C used in the current study.

Chemical Stability of Lyophilized Protein

Another important degradation route for rhuMAb HER2, deamidation and succinimide formation, may be affected by lyophilization in different sugars. Both of these reactions may be catalyzed by water and, therefore, studies were performed with a range of residual moisture levels (1–8.4% w/w) for lyophilized rhuMAb HER2 formulated at 25 mg/mL in 60 mM trehalose, 5 mM histidine, pH 6, and 0.01% polysorbate 20. During storage for 12 months at 40°C, residual moistures between 1 and 4% w/w did not have an impact on the rate or extent of loss in rhuMAb HER2 main peak (only 2% loss in main peak), as measured by IEX. At 8.4% w/w residual moisture, the rate of degradation was increased, yielding a 19% decrease in main peak after 12 months at 40°C. This deamidation may be the result of water catalysis or a reduction in the T_g of the lyophilized cake. Measurements of the frozen pH of the same formulation indicated that the pH changed from 6.3 (solution) to 6.7 on freezing to −20°C. However, the short exposure of the protein to this small pH shift is not sufficient to increase the rate of rhuMAb HER2 deamidation in the lyophilized cake.

When sugars were not present in the formulation, a loss in rhuMAb HER2 main peak in the IEX chromatogram was observed immediately after lyophilization and additional losses were observed on incubation of the lyophilized cake at 40°C (Figure 6). The addition of sucrose (Figure 6a) or trehalose (Figure 6b) reduced the rate of loss in rhuMAb HER2 main peak, and a 60 mM concentration of either was sufficient for maximal protection. Interestingly, when the sucrose/mannitol formulations were tested at these conditions, the 20 mM sucrose/40 mM mannitol formulation prevented a decrease in main peak over 3 months of storage at 40°C compared with ~1% or 5% decrease in main peak for the 60 mM sucrose or mannitol, respectively. The rate of deamidation in the 60 mM sucrose and 20:40 mM sucrose/mannitol formulations are similar, and 60 mM of sucrose or trehalose is sufficient for maximum inhibition of deamidation. Given that these formulations have a similar of retention of native protein structure in the initial lyophilized formulations, the stabilization against deamidation may be caused by maintenance of the protein in the native state.

Longer term studies at 40°C using a range of molar ratios also suggested that a specific molar ratio of sugar to protein inhibits the rate of deamidation. As shown in Table 2, high molar ratios of sugars to protein (600–1800:1) completely inhibited deamidation for 33 months at 40°C. At a 360:1 molar ratio of trehalose to protein, only a

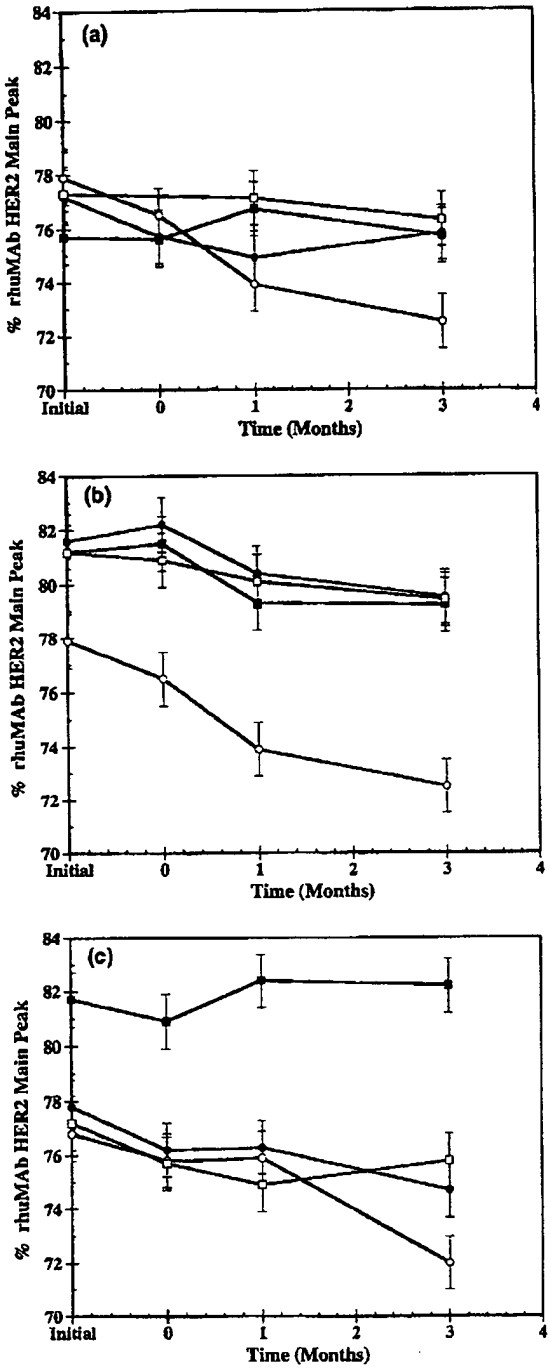

Figure 6. The rhuMAb HER2 main peak area was measured by IEX prior to lyophilization (initial) and after lyophilization with and without storage at 40°C. Formulations consisted of 5 mM histidine (pH 6) and 0.01% polysorbate 20 with 60 (closed circles), 100 (open squares), or 200 (closed squares) mM sucrose (A) or trehalose (B), or without sugar (open circles). (C) Protein in the same buffer with 60 mM mannitol (open circles), 10 mM sucrose/50 mM mannitol (closed circles), 20 mM sucrose/40 mM mannitol (closed squares), and 60 mM sucrose (open squares) was also tested.

slight decrease in main peak was observed after 51 months at 40°C. The lower molar ratio (180:1) resulted in a decrease in main peak of ~6%. The effect of sugars on stabilizing the protein against deamidation was not as significant as their effect on aggregation. Nevertheless, a specific molar ratio of sugar to protein did provide optimum long-term stability.

Each formulation studied in the aggregation and deamidation experiments was also studied for methionine oxidation, which has been previously observed for this protein.[21] After lyophilization and storage at 40°C for 3 months, there was no change in the amount of methionine oxidation (4% oxidized Fc domain), suggesting that this chemical degradation route is effectively inhibited in the lyophilized formulations.

CONCLUSIONS

These studies demonstrated that a minimal specific molar ratio of sugar to protein is sufficient to provide optimal stabilization against aggregation during storage of lyophilized rhuMAb HER2 formulations. A sugar-to-protein molar ratio of 360:1 was sufficient to inhibit aggregation and deamidation after 3 months of storage at 40°C. Both sucrose and trehalose provided equivalent protection during lyophilization and storage. Mannitol alone at the same molar ratio was only slightly less effective at preventing aggregation during storage at 40°C. Mannitol conferred stability most likely because it remained mostly amorphous during lyophilization. The addition of sucrose or trehalose to the mannitol formulations yielded equivalent storage stability compared with both sugars alone at the same total molar concentration (60 mM) and molar ratio (360:1 protein/sugar). Protein stability after storage for several years at 40°C was achieved at molar ratios of 360:1 or greater, indicating the potential for development of room-temperature stable formulations by selection of the appropriate sugar and molar ratio.

The reduction in aggregation and deamidation during storage correlated directly with inhibition of unfolding during lyophilization. Thus, it appears that the protein must be retained in its native-like state during freeze–drying to assure storage stability in the dried solid. This condition may be achieved by selecting the appropriate concentration (molar ratio) of stabilizing sugars for a particular protein.

ACKNOWLEDGMENTS

We gratefully acknowledge support from NSF Grant BES9816975. We thank Nina Le, Paul Sheehan, and Yong-sung Kim for technical assistance, and Dr. Tue Nguyen for his support of this work.

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Scand. J. Immunol. **30**, 219–223, 1989

Auto-Antibodies to Tumour Necrosis Factor α in Healthy Humans and Patients with Inflammatory Diseases and Gram-Negative Bacterial Infections

A. FOMSGAARD, M. SVENSON & K. BENDTZEN

Endotoxin Laboratory, Department of Infectious Diseases, and Laboratory of Medical Immunology, Department of Medicine TTA, Rigshospitalet University Hospital, Copenhagen, Denmark

Fomsgaard, A., Svenson, M. & Bendtzen, K. Auto-Antibodies to Tumour Necrosis Factor α in Healthy Humans and Patients with Inflammatory Diseases and Gram-Negative Bacterial Infections. *Scand. J. Immunol.* **30**, 219–223, 1989

A semi-quantitative immunoblotting method was developed to screen for serum auto-antibodies against tumour necrosis factor α (TNFα). Forty nitrocellulose strips containing identical amounts of human recombinant TNFα (rTNFα) were prepared for each set-up, and the anti-TNFα antibody immunoreactivities were scored according to the density of the resulting colour reaction. A significant number of sera from apparently healthy donors contained detectable auto-antibodies to TNFα (40%), while the strongest reaction was observed in 8%. A higher prevalence of anti-TNFα antibodies was found in sera from patients with Gram-negative bacterial septicaemia (66%), cystic fibrosis with chronic *Pseudomonas aeruginosa* lung infection (72%), and various rheumatic diseases (61%). The antibodies in sera from these patients belonged primarily to the IgG and IgM classes, the latter exhibiting the strongest response. Longitudinally collected serum samples from patients in septic endotoxin shock revealed that the anti-TNFα antibodies were induced initially during septicaemia, reaching maximum reactivities within the first week and returning to low or undetectable levels on days 9–20.

K. Bendtzen, MD, Laboratory of Medical Immunology 7544, Rigshospitalet, 20 Tagensvej, DK-2200 Copenhagen N, Denmark

The cytokine tumour necrosis factor α (TNFα) is a polypeptide derived primarily from macrophages displaying a wide variety of biological activities [2]. A potent inducer of TNFα is the lipopolysaccharide (LPS, endotoxin) of Gram-negative bacteria. Indeed, TNFα is an important mediator of endotoxic activities in Gram-negative bacterial infections, and is implicated as a prime mediator of lethal endotoxin shock [4, 5].

Antibodies to human recombinant TNFα (rTNFα) raised in rabbits inhibit the cytotoxic activity of the cytokine [7]. Moreover, TNFα antibodies have been shown to inhibit the mortality induced by systemic administration of LPS to mice [4]. These and other experiments show that anti-TNFα antibodies modify the biological effects of TNFα, and are beneficial against endotoxicosis.

We have recently reported the presence in human sera of IgG auto-antibodies to another cytokine, namely interleukin 1α (IL-1α) [11]. These auto-antibodies inhibited the binding of rIL-1α to its receptor on the murine T-cell line EL4.

We have now developed an enzyme-linked immunoblotting method to study the possible occurrence in humans of antibodies (including auto-antibodies) to TNFα. This method was used to detect auto-antibodies in sera from apparently healthy humans, and in sera of patients with acute and chronic Gram-negative bacterial infections and various rheumatic and autoimmune diseases.

MATERIALS AND METHODS

Human sera. Sera were obtained from the following donors. (1) Normal healthy male and female blood donors ($n = 50$). Age range 18–65 years. (2) Patients

RESULTS AND DISCUSSION

The transfer of rTNFα from the SDS gel to the nitrocellulose paper was ascertained by silver staining of gel pieces before and after electro-transfer [9] and by gold staining of the rTNFα on the nitrocellulose strip (Aurodye, Janssen). The position of the rTNFα band was identified by specific rabbit anti-human rTNFα antibodies in each set-up. Simultaneous running of two mini-gels resulted in 40 nitrocellulose strips available for assay. Since the strips were cut from a nitrocellulose sheet containing the same sample of rTNFα, any difference in the bands obtained after incubation with various sera could be attributed to differences in these sera.

The interassay variation of this procedure was 12% for score 0 ($n=14$), 14% for score + ($n=14$), and 9% for score ++ ($n=10$). None of the conjugates used reacted with the rTNFα band. The specificity of the human antibodies to TNFα was ascertained by absorption for 1 h at 37°C of a positive (+) serum (1:5 diluted) with 10 μg/ml rTNFα prior to the assay; in all cases, this prevented antibody binding. If the rTNFα in this solution was present as a trimer [10] it was still able to compete with the binding of the TNFα auto-antibody to the monomer of 17 kDa as presented on the nitrocellulose strip.

Fig. 1 shows the scores (0, +, ++) of the anti-TNFα serum antibody reactions. It also shows the binding of specific rabbit anti-rTNFα antibodies and the negative (control) reaction of a mixture of the different conjugates used.

Serum auto-antibodies to TNFα in individual sera

Fig. 2 shows the prevalence of naturally occurring serum auto-antibodies to TNFα in apparently healthy blood donors and the patients with various infectious and inflammatory diseases.

Most of the normal sera were negative. However, about one-third contained antibody binding to rTNFα, and 8% of the sera showed a strong anti-TNFα antibody reaction. The percentage of normal sera containing a strong anti-rTNFα reaction is similar to the percentage of normal sera found by Svenson *et al.* to contain auto-antibodies to rIL-1α [11]. However, the assay

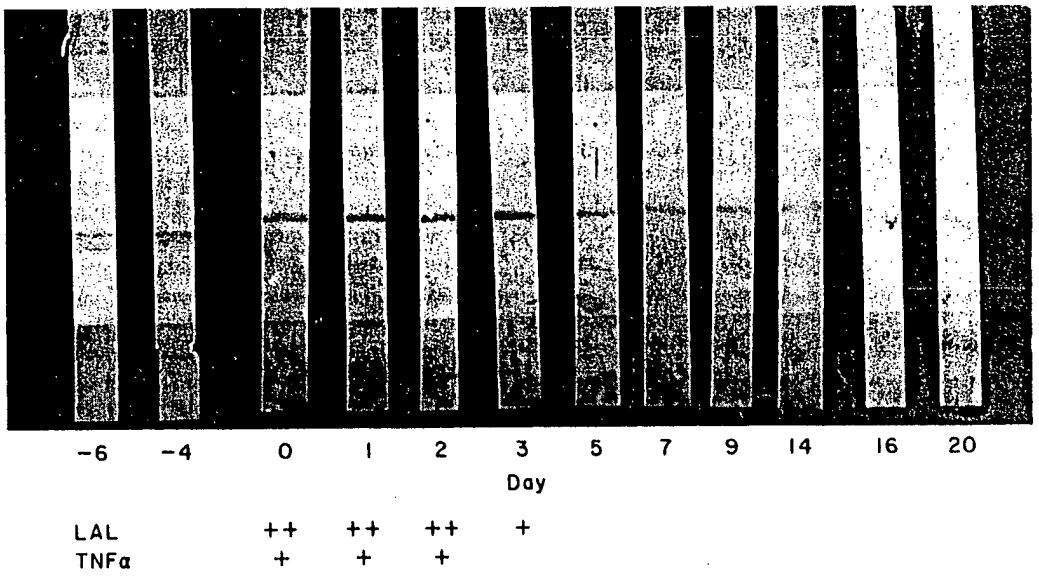

FIG. 3. Time course of appearance and disappearance of auto-antibodies to TNFα in the blood of a 77-year-old man suffering from septic shock (day 0) during recovery from pneumococcal meningitis. At this point, *Escherichia coli* were isolated from the blood and urine, and circulating endotoxin was detected on days 0–3 by a Limulus amoebocyte lysate assay (++: > 50 pg/ml; +: 5–50 pg/ml) [1]. Circulating TNFα (50–200 pg/ml) was detected by ELISA on days 0–2 [7]. The patient was in a coma and had metabolic acidosis, respiratory insufficiency, disseminated intravascular coagulation, and oliguria. He recovered after treatment with antibiotics and artificial respiration.

and IgG immunoglobulin classes. These anti-TNFα antibodies were primarily found in patients with signs of Gram-negative bacterial infections but also in increased amounts in sera of patients with various rheumatic diseases. The biological relevance of serum auto-antibodies to TNFα is unclear, but they may play important roles in regulative and/or protective functions. For example, anti-TNFα antibodies have been shown to protect mice against lethal effects of systemically administered rTNFα or LPS [4]. It may therefore be speculated that auto-antibodies to TNFα may exhibit similar protective functions in mice as well as humans.

The immunoblotting method described here seems suitable for the screening of anti-TNFα auto-antibodies, and the same technique might be used for the study of auto-antibodies to other cytokines.

ACKNOWLEDGMENTS

We thank Dr Niels Høiby, Department of Clinical Microbiology and Dr Henrik Permin, Department of Rheumatological Diseases, Rigshospitalet, University Hospital, Copenhagen, for provision of sera from cystic fibrosis patients and patients with rheumatic diseases. Ms Susanne Jensen provided excellent technical assistance. The study was supported by the Danish Association against Rheumatic Diseases, The Danish Medical Research Council, and the Danish Biotechnology Programme.

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Received 10 February 1989
Accepted in revised form 3 April 1989

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ELSEVIER

International Journal of Pharmaceutics 237 (2002) 57–69

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

Protection mechanism of Tween 80 during freeze–thawing of a model protein, LDH

Anna Hillgren [a], Jan Lindgren [b], Maggie Aldén [a,*]

[a] *Department of Pharmacy, Physical and Inorganic Pharmaceutical Chemistry, Uppsala University, Biomedical Center, P.O. Box 580, S-751 23 Uppsala, Sweden*

[b] *Department of Materials Chemistry, The Ångström Laboratory, Uppsala University, P.O. Box 538, S-751 21 Uppsala, Sweden*

Received 19 September 2001; received in revised form 11 January 2002; accepted 14 January 2002

Abstract

The purpose of the study was to investigate the protective mechanism of a non-ionic surfactant, Tween 80, at freeze–thawing with controlled temperature history of a model protein, lactate dehydrogenase (LDH). The system was examined by differential scanning calorimetry (DSC) and infrared spectroscopy (IR). LDH activity assays were performed spectrophotometrically. In all samples, independent of temperature history and addition of surfactant, all water was crystallized to polycrystalline ice at temperatures below −20 °C. The size and perfection of the ice crystals could be varied by a range of cooling rates giving different degrees of undercooling. At Tween concentrations below the cmc at crystallization, lower concentrations were required at low cooling rates compared to higher cooling rates to protect LDH. Concentrations above cmc of Tween reduced the protection at a cooling rate of 5 °C min^{-1} and at quenching in $N_2(l)$. The amount of Tween needed for complete protection correlated to the surface area of the ice crystals at a certain temperature history. Tween 80 protects LDH from denaturation at freeze–thawing by hindering its destructive interaction with the ice crystals. The protective effect might be obtained when Tween molecules compete with the protein for sites on the ice surface. The optimum concentration of Tween needed for complete protection is dependent on the temperature history.

Keywords: Tween 80; LDH; Freeze–thawing; Temperature history; DSC; IR spectroscopy

1. Introduction

Protein instability in aqueous solution is a major difficulty in producing pharmaceutical formulations, therefore proteins are often freeze-dried to increase their stability. Freezing is an important step in this process, but it induces several stresses capable of protein denaturation. There are a wide variety of protective excipients added to the formulation, such as sugars, amino acids, polymers and nonionic surfactants. It has been found that low concentrations, below the critical micelle concentration (cmc) of nonionic surfactants, such as polyoxyethylene 20 sorbitan monooleate (Tween 80) provide a high degree of protection (Nema and Avis, 1993; Carpenter et al., 1997).

* Corresponding author. Tel.: +46-18-471-4369; fax: +46-18-471-4377.

E-mail address: maggie.alden@farmaci.uu.se (M. Aldén).

0378-5173/02/$ - see front matter
PII: S0378-5173(02)00021-2

There are several possible mechanisms suggested in the literature for protein protection by surfactants. One mechanism is specific binding/interaction with hydrophobic sites on the protein, where Tween can bind weakly to the protein surface (Bam et al., 1995; Katakam et al., 1995; Bam et al., 1998). The protective ability may then correlate with the amount of surfactant needed to saturate these hydrophobic patches, that is the molar ratio of surfactant to protein. The interaction between Tween and LDH in the system used here is very weak at room temperature, as was determined by steady-state and time-resolved fluorescence spectroscopy and diffusion NMR experiments (Hillgren et al., 2002).

Another possible mechanism of protection is a situation where the additive is preferentially excluded from the surface of the protein. This would create a thermodynamically unfavourable system, especially if the protein is denatured, because the surface area exposed to the solvent increases with protein unfolding. The most stable system, the native structure, is thus favoured (Timasheff, 1982; Carpenter et al., 1991). Polyethylene glycol (PEG) is supposed to protect proteins by this mechanism (Carpenter et al., 1991). Surfactants are, however, generally operative at concentrations where volume exclusion effects can be neglected.

A third theory is that denaturation occurs at the ice-water interface. Rapid cooling leads to formation of many small ice crystals, with larger surface area than the crystals produced by slow cooling. It has been shown in previous studies that fast cooling provides much more stress to proteins than a low cooling rate (Chang et al., 1996; Strambini and Gabellieri, 1996; Jiang and Nail, 1998; Kerwin et al., 1998; Kreilgaard et al., 1998). Surfactants may stabilize protein structure by competing with the protein for adsorption on the ice-water interface.

The stability of a protein is also dependent on its own concentration. A concentrated protein solution is more resistant to freeze-induced denaturation. This intrinsic stability may be due to damage and denaturation of the protein at the ice-water interface during freezing. If a finite number of protein molecules can be denatured at this interface, then increasing concentration will lead to a smaller percentage of damaged molecules (Carpenter et al., 1997). It is a well known fact that protein can be adsorbed to many surfaces and interfaces (Claesson et al., 1995) and several studies have also shown that different proteins are destabilized or have changed their secondary structure due to surface adsorption (Wang, 1999).

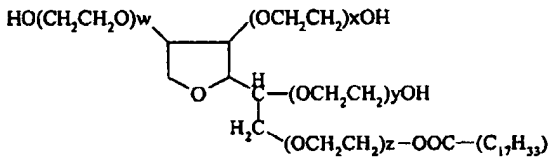
The purpose of this study was to further investigate the ice-water interface theory as a possible mechanism for protein protection by nonionic surfactant at low temperature. A controlled temperature history was used in the experiments by DSC and IR spectroscopy to investigate the ice aggregation state and the ice crystal size and energy. Lactate dehydrogenase (LDH) was used as a model protein in both low concentrations to avoid self-stabilization and in high concentrations. The effect of different concentrations of Tween 80 on the protein protection during the freeze-thawing process in relation to ice surface was also evaluated.

2. Experimental

2.1. Materials

Lactate dehydrogenase (LDH) from rabbit muscle, crystalline suspension in 65% saturated $(NH_4)_2SO_4$ solution, pH 7.2 (ICN Pharmaceuticals Inc., USA). Polyoxyethylene 20 sorbitan-monooleat (Tween 80) (Kebo, Sweden). Nicotinamide adenine dinucleotide reduced form (NADH) in preweighed vial, 0.2 mg and sodium pyruvate solution 22.7 mM, pH 7.5 (Sigma, USA). Sodium phosphate buffer 0.1 M, pH 7.5. Sodium citrate buffer 10 mM, pH 6.5.

Tween 80:



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2.2. Methods

2.2.1. Preparation of solutions

Prior to experiments, the LDH suspension was dialyzed against 10 mM sodium citrate buffer, pH 6.5. Citrate buffer was selected because it has minimal pH change during freezing (Carpenter et al., 1997). The dialyzed LDH was concentrated during centrifugation using Microsep Centrifugal Concentrators (Pall Filtron Co., USA) and the concentration of the enzyme was determined spectrophotometrically with a Spectronic Genesys spectrophotometer (Milton Roy Co., USA). The UV absorbance at 280 nm was linearly related to concentration in the range of 0.1–1.1 mg ml^{-1}.

2.2.2. Assay of enzyme activity

LDH activity was measured spectrophotometrically with a Spectronic Genesys spectrophotometer (Milton Roy Co.). The 1.44 ml reaction mixture contained 55 μM NADH in 0.1 M phosphate buffer, pH 7.5 and an aluminium pan with 20 μl LDH sample (25 μg ml^{-1}). The enzymatic reaction was started by adding sodium pyruvate (1.9 mM) and monitored by measuring the decrease in absorbance at 340 nm. The recovered activity of a frozen LDH sample was calculated as percentage of the activity of an identical unfrozen sample that had been stored in an aluminium pan. The activities are presented as mean values with the S.D. based on three determinations.

2.2.3. Differential scanning calorimetry (DSC)

The solutions were examined using a DSC 220C oscillating differential scanning calorimeter (Seiko Instruments Inc., Japan) equipped with the Exstar6000 software, version 3.4A. The samples were kept in non-sealed aluminium pans in an atmosphere of nitrogen. The calorimeter was temperature- and heat-calibrated with indium, tin, gallium and mercury as standards. The temperature history included freezing from 30 to −60 °C and then heating to 30 °C with a controlled cooling rate between 0.5 and 90 °C min^{-1} and a heating rate of 5 °C min^{-1}. The concentrations were 25 μg ml^{-1} LDH and 0.23 μg ml^{-1} to 3 mg ml^{-1} Tween 80 in 10 mM sodium citrate buffer, pH 6.5.

Thermograms were recorded at both cooling and heating. The results are presented as mean values with the S.D. based on three determinations.

2.2.4. Infrared spectroscopy

Infrared spectra were collected using a Fourier transform infrared (FTIR) spectrometer, BioRad FTS-45 (Digilab Division, USA). The protein solutions were placed in an IR cell with CaF_2 windows. Water and carbon dioxide vapour was removed by purging the spectrometer with nitrogen gas. The resolution was 2 cm^{-1} and all spectra were collected by 16 scans and the average was calculated. The temperature was 20–60 °C, obtained in a variable temperature cell using $N_2(l)$ as cooling agent. The concentrations were 20 mg ml^{-1} LDH and 0.186–267 mg ml^{-1} Tween 80 in 10 mM sodium citrate buffer, pH 6.5, in the absence or presence of 5% D_2O. Spectrum without D_2O was subtracted from spectrum with D_2O and the OD-stretching mode of isotopically isolated HDO molecules at ≈ 2500 cm^{-1} were studied.

To study the secondary structure, spectral features arising from water vapour and buffer components were subtracted and the second-derivative spectra were calculated. To determine the similarity between two second-derivative spectra, the correlation coefficient, r, was calculated.

$$r = \frac{\sum x_i y_i}{\sqrt{\left(\sum x_i^2 \sum y_i^2\right)}}$$

In the equation, x_i and y_i are the spectral absorbance values of the reference and sample spectra at the ith frequency position (Prestrelski et al., 1993). The correlation coefficient between two spectra of a given protein equals 1 when there is no conformational change in the protein. The larger the change in conformation, the greater the differences between the spectra and the smaller the value of r.

2.2.5. Surface tension measurements

The surface tension measurements were performed by a du Noüy tensiometer (Krüss Optisch-

Mechanische Werkstätten, Germany) against air at 25–3 °C. All measurements were performed at ≈10–15 min after formation of a new surface (Persson, 1999). Surface tension (γ) of pure water was also measured at different temperatures and used to correct the measurements of Tween solutions. The concentrations were 0.5–0.0001% w/v (0.76 μM–3.8 mM) of Tween 80 in Milli Q water. The critical micelle concentration (cmc) of Tween 80 at different temperatures was obtained from plots of surface tension versus the logarithm of the concentration.

The surface area occupied per Tween molecule was calculated from the surface excess concentration, Γ, through the Gibbs equation (Jönsson et al., 1998).

3. Results and discussions

3.1. Behaviour of the components in the system at low temperature

3.1.1. Crystallization of ice

To examine the aggregation state of water and ice, the OD-stretching mode of HDO molecules in an IR spectrum was followed. This vibration occurs at ≈2500 cm^{-1}. The wavenumber and the size of the peak are dependent on the environment of the OD-bond. It moves to a lower wavenumber and becomes narrower as the crystallinity increases (Franks, 1982). In water, the environment is amorphous and the mobility of water molecules is large. The OD-stretching mode then corresponds to a broad peak at 2500 cm^{-1} (Fig. 1). In polycrystalline ice, the vibration frequency decreases to ≈2430 cm^{-1} and the band becomes narrower. The peak positions are also dependent on the temperature, both before and after the crystallization, but the full width at half maximum (FWHM) is independent of the temperature (Franks, 1982).

In our experiment, the peak position decreases as the temperature decreases with a sharp change when the ice crystallizes at about −15 °C (Fig. 2a). The addition of Tween 80 and LDH made no significant difference to this behaviour. The OD-stretching band for amorphous solid water is found at ≈2450 cm^{-1} and for polycrystalline ice, ≈2430 cm^{-1} (Franks, 1982). At temperatures below −20 °C there is no liquid water or amorphous solid water detected by IR, only polycrystalline ice. If the cooling rate increased from 1 to 10 °C min^{-1}, there was still no detectable liquid

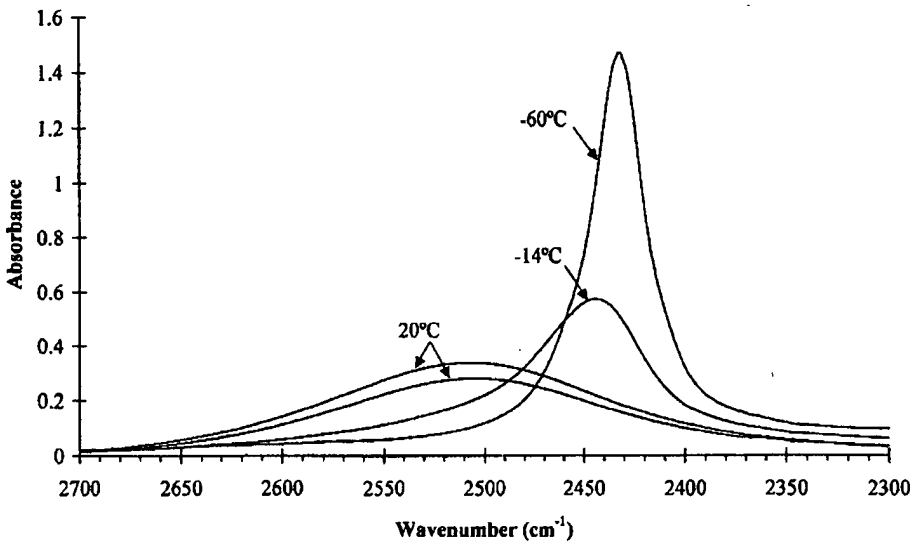

Fig. 1. The OD-stretching obtained by FTIR for sodium citrate buffer at different temperatures. There are two spectra at 20 °C, one before and one after freeze–thawing.

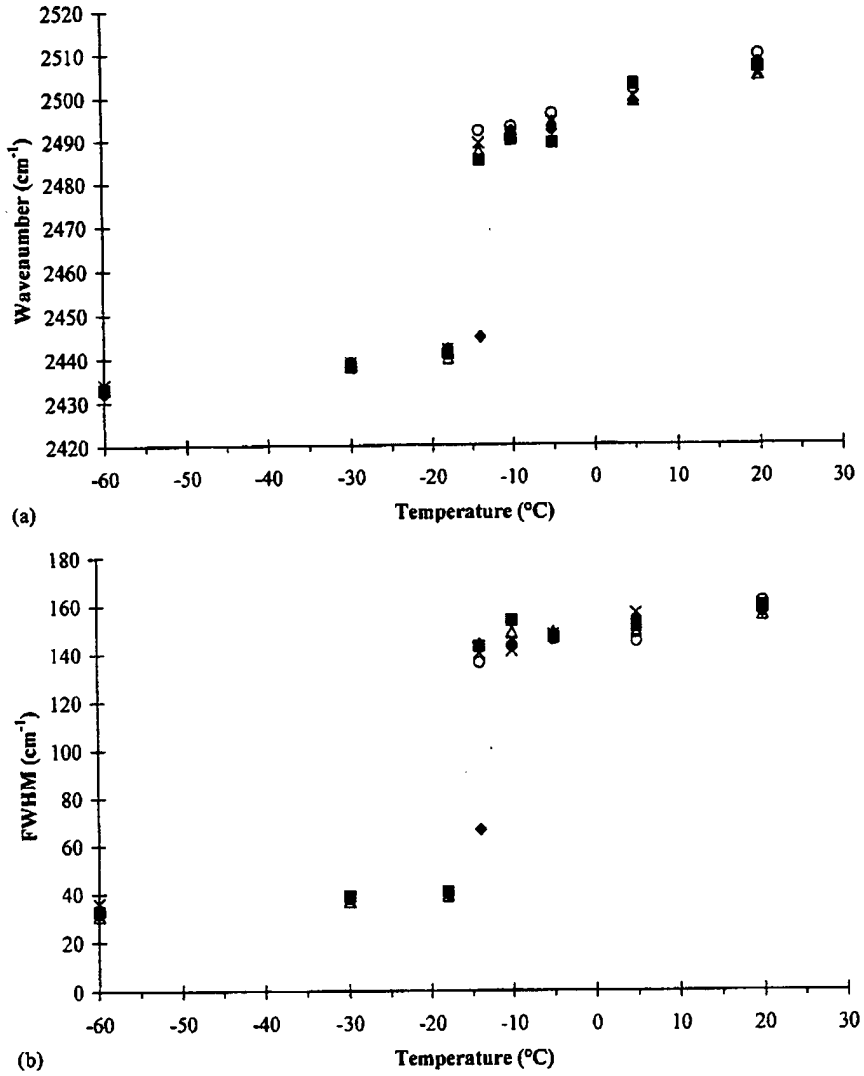

Fig. 2. The change of wavenumber (a) and the full width at half maximum FWHM (b) during cooling for the OD-stretching transition. (◆) Citrate buffer, (■) LDH 20 mg ml^{-1}, (□) LDH 20 mg ml^{-1} and Tween 80 0.186 mg ml^{-1}, molar ratio 1:1, (×) LDH 20 mg ml^{-1} and Tween 80 26 mg ml^{-1}, molar ratio 1:146 and (○) Tween 80 267 mg ml^{-1}.

water or amorphous solid water, only one single, symmetrical peak in the spectrum at 2434 cm^{-1} (data not shown). The FWHM, for both water and ice, is independent of the temperature and the sharp decrease between -10 and -20 °C reflects the crystallization (Fig. 2b). The increased cooling rate, from 1 to 10 °C min^{-1}, did not change this behaviour (data not shown).

The experiments thus show that regardless of cooling rate and addition of Tween 80 and/or LDH, all water is crystallized to polycrystalline ice below -20 °C.

3.1.2. Denaturation of LDH

Denaturation of proteins can be monitored with different techniques. IR spectroscopy is one powerful method to study structural changes in any state of a protein, i.e. aqueous, frozen or dried. Nine characteristic vibrational bands or group frequencies that arise from the amide group

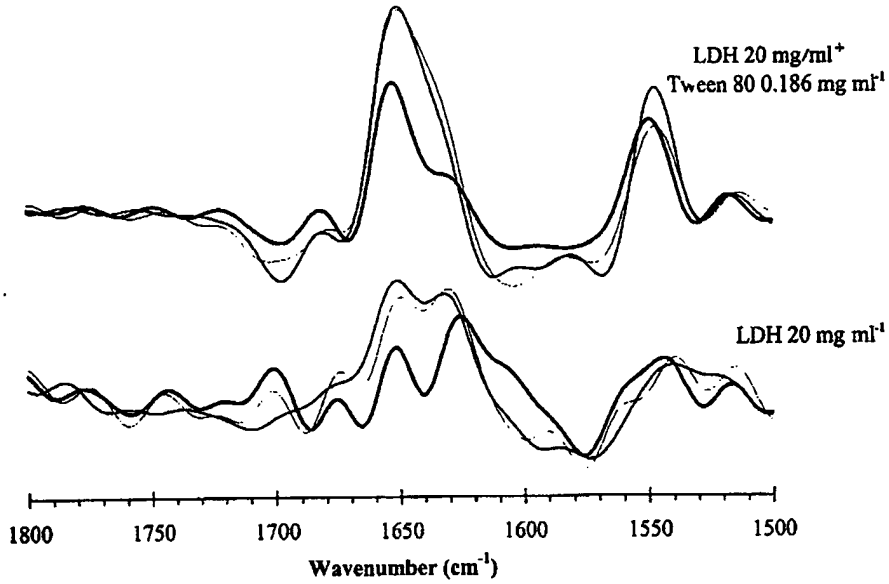

Fig. 3. Second derivative FTIR spectra plotted as the absolute values of 20 mg ml^{-1} LDH with or without Tween 80 at different temperatures. ——, 20 °C; ——, −60 °C; ——, 20 °C after one freeze–thawing cycle.

of protein have been identified. The amide I band (1700–1620 cm^{-1}) is almost entirely due to the C = O-stretching vibration of the peptide linkages. This is the most studied band for determination of secondary structures, since it is sensitive to small variations in molecular geometry and hydrogen bonding patterns within the protein (Dong et al., 1995).

Fig. 3 shows the second-derivative IR spectra for LDH and LDH with the addition of Tween 80 at different temperatures. Each type of secondary structure gives a different C = O-stretching frequency in the spectrum. Strong bands near 1656 and 1636 cm^{-1} arise from α-helical and β-sheet structures, respectively (Prestrelski et al., 1993). The correlation coefficient, r, defined in Section 2, is used to determine similarities between different spectra. If there is no change in the conformation of the protein, the correlation coefficient equals 1 and becomes smaller with greater changes in the protein structure.

The spectrum of 20 mg ml^{-1} LDH at −60 °C is altered relative to the aqueous spectrum at 20 °C, with a correlation coefficient of 0.609 (Table 1). This indicates that freezing significantly changes the conformation of the protein. As the sample is thawing, the conformation seems to recover, with an r-value of 0.968 between LDH at 20 °C before and after freeze–thawing (Table 1). Relatively high concentrations of protein are required to obtain an IR spectrum of high quality and it is well known that increasing protein concentration leads to increased resistance to denaturation during freezing (Chang et al., 1996; Carpenter et al., 1997). We found that the recovered activity of pure LDH after freezing to −60 °C with identical temperature history varied with concentration and was 45% at 25 μg ml^{-1},

Table 1
The correlation coefficient (r) between two second derivative IR spectra of 20 mg ml^{-1} LDH and 0.186 mg ml^{-1} Tween 80 at different temperatures

Reference spectrum	Sample spectrum	r
LDH at 20 °C	LDH at −60 °C	0.609
LDH at 20 °C	LDH at 20 °C	0.968
LDH at 20 °C	LDH+Tween 80 at 20 °C	0.888
LDH+Tween 80 at 20 °C	LDH+Tween 80 at −60 °C	0.963
LDH+Tween 80 at 20 °C	LDH+Tween 80 at 20 °C	0.992

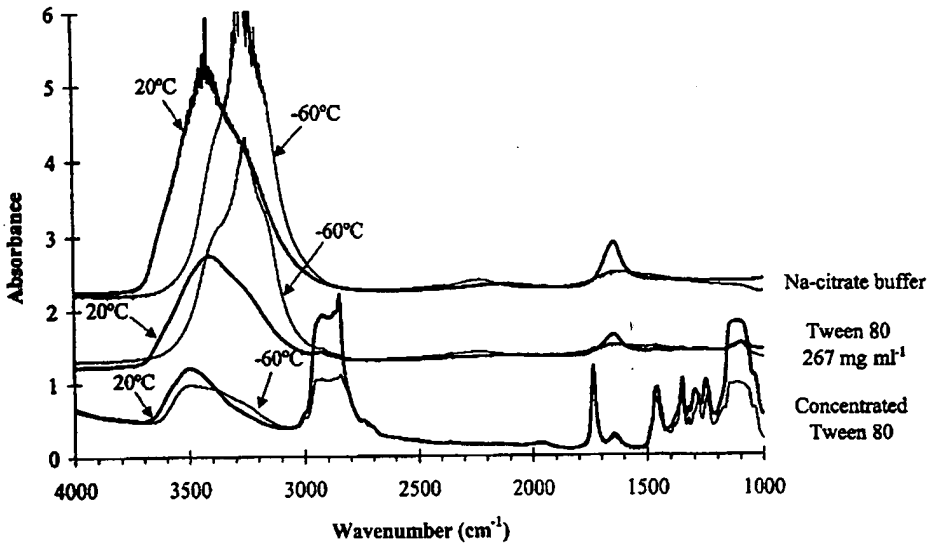

Fig. 4. FTIR spectra for different samples with Tween 80 and sodium citrate buffer at 20 and −60 °C, respectively.

63% at 0.1 mg ml^{-1} and 103% at 10 mg ml^{-1}, respectively. Thus, concentrations of 20 mg ml^{-1} protein, which were used in the IR experiment, do not denature if the complete freeze–thawing process is considered, although some conformational changes seem to occur at low temperatures.

Addition of Tween alters the conformation of LDH at room temperature (Table 1), indicating an interaction between LDH and Tween 80 at the high concentrations used here. The IR spectrum of LDH with the addition of Tween at −60 °C is only slightly changed compared to the spectrum of the same solution at room temperature. The conformation of LDH is also preserved during thawing with an r-value of 0.992 of this solution before and after freeze–thawing (Table 1). This shows that addition of Tween is hindering the denaturation of the protein during a freeze–thawing process.

3.1.3. Phase transitions of Tween 80

To be able to understand the cryoprotective effect of Tween 80 in protein solutions, it is of great importance to have some knowledge about the behaviour of the aqueous solution at low temperature. The cmc of Tween 80 in water at room temperature is, according to the literature, 10 μM (Sivars and Tjerneld, 2000) or 11 μM (Wan and Lee, 1974). In this study, the cmc in water was determined to be ≈13 μM. In a sodium citrate buffer solution at room temperature the cmc was 10–30 μM and the addition of LDH increased the cmc, as was determined in this laboratory by fluorescence spectroscopy and NMR (Hillgren et al., 2002). For nonionic surfactants, the cmc increases with decreasing temperature (Jönsson et al., 1998). If the temperature is decreasing to −3 °C, the cmc of Tween 80 in water increases to 130 μM, according to our experiments (data not shown). Measurements at lower temperatures were difficult to perform because of the freezing of the solution.

The IR spectra of different concentrations of Tween 80 in sodium citrate buffer and of pure buffer solution are shown in Fig. 4. Concentrated Tween, which may include up to 3% moisture, shows characteristic bands of OH-stretching (3600 cm^{-1}), CH2-stretching (2900 cm^{-1}) C=O-stretching (1750–1735 cm^{-1}) and HOH-bending (1600 cm^{-1}). When water is crystallized the OH-stretching vibration mode, reflecting both water and Tween OH-groups, changes from 3600 to ≈3300 cm^{-1} (Franks, 1982). The intensity of the HOH-bending mode decreases rapidly as the hydrogen bond strength of the molecular environment increases, e.g. when water freezes to ice

(Devlin, 1990). As concentrated Tween is freezing, the OH-stretching mode is slightly shifted to a lower wavenumber, but the intensity of the HOH-bending mode is unchanged. In a sample with pure buffer or diluted Tween, both the wavenumber of the OH-stretching and the intensity of the HOH-bending are changed (Fig. 4). This difference indicates that in concentrated Tween 80, the water forms a structure different from water and ice when the temperature is decreasing. In a previous study, DSC measurement of a concentrated solution of Tween 80 showed a phase transformation at about −14 °C (Hillgren et al., 2002) that could indicate the presence of such a structure formed by Tween and ice/water at low temperature. Since Tween has a certain amount of side chains of polyethylene, the transition might reflect melting of a structure similar to the PEG-ice structure, proposed to be a hydrate (Antonsen and Hoffman, 1992), melting at about −15 °C (Aldén and Magnusson, 1997).

In the following experiments, however, the Tween concentrations are low enough to avoid the creation of such a structure and both OH-stretching and HOH-bending follow the pattern of pure water.

3.2. The ice-formation at the freeze–thawing process and recovered activity of LDH

3.2.1. Influence of cooling rate on ice crystal formation

To investigate the protective ability of Tween 80 in the freeze–thawing process while the size of ice crystals and thus the amount of ice-water interface is varied, a series of DSC experiments were performed. A low concentration of LDH (25 μg ml^{-1}) was used to avoid intrinsic stability of the protein. At this concentration, the protein is more or less denatured during a freeze–thawing process (Aldén and Magnusson, 1997). The size of the ice crystals was varied by selecting a cooling rate between 0.5 and 90 °C min^{-1} in the DSC experiments and a cooling rate of about 10^2–10^3 °C min^{-1} when the sample was frozen instantly in liquid nitrogen. The sample could not, however, immediately follow a cooling rate larger then 20 °C min^{-1} in the DSC experiments, therefore the results presented in this study refer to strictly controlled cooling rates of 0.5–20 °C min^{-1}. The thermogram of 25 μg ml^{-1} LDH in citrate buffer is shown in Fig. 5, where the cooling

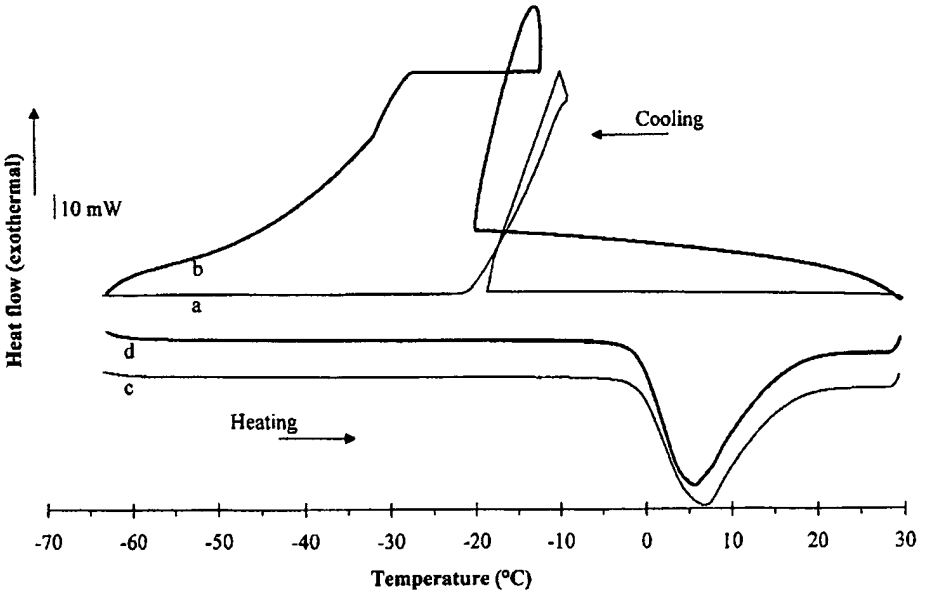

Fig. 5. Cooling and heating thermograms obtained by DSC for 25 μg ml^{-1} LDH in sodium citrate buffer with a heating rate of 5 °C min^{-1}. (a) Cooling process with a cooling rate of 1 °C min^{-1} and (c) the corresponding heating process. (b) Cooling process with a cooling rate of 20 °C min^{-1} and (d) the corresponding heating process.

Table 2
Heat of transformation and peak temperatures for 25 μg ml^{-1} LDH with 0.23 μg ml^{-1} Tween 80, molar ratio 1:1 and different cooling rates

CR (°C min^{-1})	Crystallization				Melting			
	T_{onset} (°C)	S.D.	ΔH (J g^{-1})	S.D.	T_{onset} (°C)	S.D.	ΔH (J g^{-1})	S.D.
0.5	−17.2	0.2	−257.7	4.1	−0.9	0.1	286.9	5.4
1	−15.4	1.3	−267.2	2.2	−0.8	0.2	301.5	3.3
5	−17.1	1.6	−268.7	2.8	−0.7	0.1	306.2	3.0
20	−21.5	1.3	−174.8	11.9	−0.8	0.1	307.6	9.3

rates were 1 and 20 °C min^{-1}, respectively and the heating rate in both cases was 5 °C min^{-1}. The thermograms show a crystallization peak at −10 °C to −20 °C and a melting peak at 5–6 °C. It also illustrates the complex temperature variation at the crystallization, since the sample temperature increases by several degrees as the crystallization process starts and before equilibrium is reached in the cooling process.

Table 2 shows the heat of transformation and peak temperatures for LDH and Tween 80 at molar ratio 1:1. There were no significant differences in the values between this composition of the sample and the other samples. The onset temperature for the crystallization decreased when the cooling rate increased to 20 °C min^{-1}, indicating a more undercooled solution. The heat of crystallization also decreased with increased cooling rate to 20 °C min^{-1}. This shows that the less sample transformed to solid state, the less amount of ice is formed or that the structure that is formed contains more energy. Since IR spectroscopy showed that regardless of cooling rate all water crystallizes to polycrystalline ice and no amorphous phase is formed, the decrease in ΔH for the crystallization must then depend on the size and perfection of the ice crystals. The crystallization temperature affects the size of ice crystals. As the cooling rate increases, the crystallization temperature decreases, the solution becomes more undercooled and the nucleation rate increases. This means that more nuclei of ice are created and thereby a larger amount of small crystals (Michelmore and Franks, 1982; Bronshteyn and Steponkus, 1995). A high cooling rate thus creates smaller and more imperfect ice crystals, giving a higher energy of the system than a low cooling rate. During slow freezing, the system is less undercooled, fewer nuclei of ice are formed and the crystals have time to grow and become more perfect. The same amount of ice in the shape of small crystals gives a much larger surface area than larger ones, which means more opportunity for the ice crystals to damage the protein.

The heat of melting and the onset temperature of melting were, however, not affected by the cooling rate. The same amount of ice with the same energy content melts regardless of cooling rate. This confirms that the crystallization and growth of crystals continues during the entire cooling process and also during heating at low temperatures (Bronshteyn and Steponkus, 1995).

3.2.2. The amount of Tween 80 needed for protection at different cooling rates

The recovered activity of LDH in solution without the addition of Tween was ≈50% after freezing with a cooling rate of 1–5 °C min^{-1}. It decreased to some extent with a cooling rate of 20 °C min^{-1} and was only 4% when the sample was frozen instantly, by direct insertion in liquid nitrogen (Table 3).

When Tween 80 was added in a certain concentration to the protein solution, the protective effect varied with the cooling rates that was used. The addition of 0.23 μg ml^{-1} (0.17 μM) Tween 80, molar ratio 1:1, did not provide protection of LDH at high cooling rates (Table 3). Tween–LDH interactions are very weak at the molar ratio and temperatures used here, as found previously by fluorescence spectroscopy (Hillgren et al., 2002) and are obviously not sufficient for

protection. The recovered activity increased however compared to the formulation without Tween 80 when the cooling rate decreased to 1 °C min^{-1}. A cooling rate of 0.5 °C min^{-1} with a larger undercooling (Table 2) decreased the activity again. The addition of 33 μg ml^{-1} (25 μM) Tween 80, molar ratio 1:146, offered complete protection at cooling rates of 1–20 °C min^{-1} (Table 3). When the sample was frozen in liquid nitrogen, the recovered activity increased from 4% in pure buffer to 25% when Tween 80, molar ratio 1:146 was added.

Table 4 illustrates the recovered activity of LDH in a low concentration with different concentrations of Tween 80 added at a certain temperature history. The sample was frozen with a cooling and a heating rate of 5 °C min^{-1} or instantly frozen in liquid nitrogen and thawed at room temperature. The lowest concentration of Tween, 0.17 μM, did not protect LDH during freeze–thawing. At the lower cooling rate, 25 μM Tween 80 provided full protection and at higher concentrations, above cmc at crystallization, the protection was again reduced (Table 4). With a very high cooling rate, LDH was almost completely protected with the addition of 76 μM Tween 80, but the protection decreased at concentrations above cmc of Tween. The minimum amount of Tween needed for complete protection was thus dependent on the cooling rate and thereby the degree of undercooling and hence on the size of the ice crystals and the amount of ice surface.

3.2.3. Ice surface and amount of Tween 80

If Tween 80 is protecting LDH by reducing the interaction between protein and ice, there should be a correlation between the amount of Tween needed for protection at crystallization and the created ice surface during a specific freeze–thawing cycle. Nonionic surfactants have a strong temperature dependence of the adsorption on surfaces. The area occupied per Tween molecule, calculated from surface tension measurements (data not shown), increased as the temperature decreased. At room temperature, the occupied area of one Tween molecule is ≈ 60 $Å^2$ and at temperatures below −3 °C, the area per molecule is larger than 80 $Å^2$. The minimum radius of ice crystals completely covered with Tween monomers at different Tween concentrations is calculated in Table 4. The assumptions made for the calculations are that all ice crystals are perfect spheres, that the area occupied by one Tween monomer at crystallization is 80 $Å^2$, that Tween exist as monomers at all concentrations (which of course is not true over cmc) and that the density of ice is 0.92 g cm^{-3}. At the concentration where Tween gives full protection at low cooling rates (25 μM), the molecules can cover the surface of crystals with a radius of minimum 268 μm. Some 76 μM Tween 80, which provides almost complete protection at both low and high cooling rates, can cover ice-crystals with a minimum radius of 89 μm. According to Evans et al. (1996), ice crystals have a diameter of ≈ 100–200 μm if a freezing rate of up to 3 °C min^{-1} is used.

Table 3
The recovered activity of 25 μg ml^{-1} LDH with addition of different molar ratio of Tween 80 during freeze–thawing at different cooling rates, CR and constant heating rate, 5 °C min^{-1}

CR (°C min^{-1})	LDH		LDH + Tween 80 0.23 μg ml^{-1} 1:1		LDH + Tween 80 33 μg ml^{-1} 1:146	
	Activity (%)	S.D.	Activity (%)	S.D.	Activity (%)	S.D.
0.5			54.6	4.8		
1	50.7	7.6	68.4	2.9	99.0	12.8
5	52.3	5.5	51.9	4.6	93.4	7.5
20	40.4	5.5	45.3	8.8	89.3	6.8
$N_2(l)$	4.3	0.7	4.4	1.1	25.5	0.9

Table 4
Recovered activity of 25 μg ml^{-1} LDH after freeze-thawing with different cooling rates and concentrations of Tween 80

Tween 80		Molar ratio LDH:Tween	CR = 5 °C min^{-1}		Frozen in N_2(l)		Minimum radius of ice crystals completely covered with Tween molecules
			Activity (%)	S.D.	Activity (%)	S.D.	
0	0	–	52.3	5.5	4.3	0.7	
0.17 μM	0.23 μg ml^{-1}	1:1	51.9	4.6	4.4	1.1	3.8 cm
25 μM	33 μg ml^{-1}	1:146	93.4	7.5	25.5	0.9	268 μm
76 μM	0.1 mg ml^{-1}	1:447	80.0	4.9	89.9	12.6	89 μm
0.23 mM	0.3 mg ml^{-1}	1:1340	58.8	9.7	66.9	1.5	30 μm
2.23 mM	3 mg ml^{-1}	1:13398	78.0	–	58.4	2.3	3.0 μm

The results obtained here indicate that the cooling rate of 5 °C min^{-1} used in this study creates ice crystals of similar size.

3.3. The protection mechanism

A possible explanation for the concentration dependence of the protective ability of Tween 80 could be that denaturation of proteins occurs when the protein adsorbs to the ice crystal surface in the crystallization process. Tween can protect LDH by competing with the protein for sites on the ice surface, as is schematically illustrated in Fig. 6. At very low concentrations of Tween there are not enough Tween molecules to cover the ice surface and hence Tween cannot fully protect LDH from denaturation. If the concentration of Tween increases, but is still below the cmc at crystallization, the opportunity of the protein to interact with the ice crystals is reduced. When the amount of Tween molecules is large enough to cover the ice surface completely, LDH is fully protected from denaturation. If the Tween concentration increases further to about the cmc at crystallization temperature, there exist both micelles and monomers in equilibrium. Depending on the kinetics of micelle formation, the shape of the micelles and their affinity to different interfaces, there might be a distribution of micelles and monomers where part of the ice surface is uncovered and destructive interactions can occur again with the protein. As the concentration increases far above cmc at crystallization temperature, more micelles are formed and the ice surface

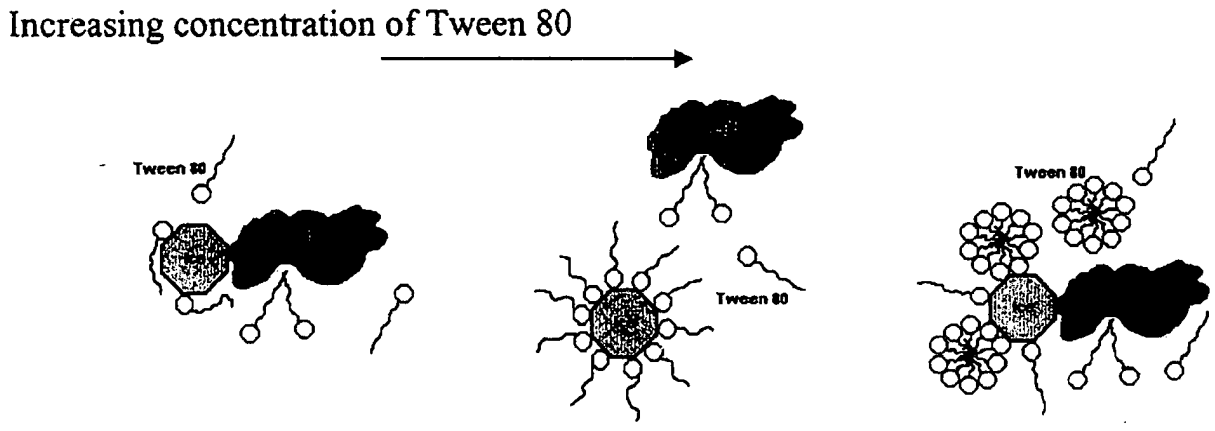

Fig. 6. Illustration of possible interactions between Tween 80, ice and LDH in the solid state at varying concentrations of Tween 80.

might be more or less covered. Since the ice surface area is correlated to the cooling rate and the undercooling of the system, a higher concentration of Tween 80 is needed for complete protection of the protein when the cooling rate is enhanced.

4. Conclusions

During the freeze–thawing process with the same temperature history, very low concentrations of Tween 80 cannot protect LDH in low concentrations but higher concentrations of Tween, below cmc at crystallization, have full protective effect. With an increasing freezing rate, a higher concentration of Tween 80 is needed for full protection. These results suggest a protective mechanism of Tween that supports the ice-water theory, since only a weak interaction exists between Tween and LDH, not large enough for protection. At temperatures below −20 °C, only polycrystalline ice is present in the system. A low cooling rate at crystallization creates larger and more perfect ice crystals than higher rates. Since the ice surface seems to be a damaging environment for the LDH molecules, Tween 80 can protect LDH from denaturation by hindering its interaction with the ice. A varying amount of Tween molecules can cover the ice crystal surface totally or partly and thus reduce the possible protein–ice interaction. The amount of Tween that is needed for full protection at a certain temperature history correlates to the area of the ice crystals.

Acknowledgements

The authors are very grateful to Dr Hans Evertsson for valuable discussions. Financial support from the Swedish Research Council for Engineering Sciences is gratefully acknowledged.

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Ann Rheum Dis 1999;58:(Suppl I) I70–I72

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Preliminary results of early clinical trials with the fully human anti-TNFα monoclonal antibody D2E7

Joachim Kempeni

Current pharmacological treatments for rheumatoid arthritis (RA), including non-steroidal anti-inflammatory drugs (NSAIDs), disease modifying anti-rheumatic drugs (DMARDs), and corticosteroids, have been moderately successful in alleviating the discomforts associated with swollen, painful joints. However, conventional medical approaches to treatment have had little or no impact on the disease course of RA.[1] Innovative strategies, particularly those based on new concepts in the immunobiology of RA, are being developed to target cellular inflammatory mechanisms and potentially prevent disease progression. The more promising of these treatments seem to be those that block the effects of tumour necrosis factor (TNF) α, because this proinflammatory cytokine seems to play a central part in the immunopathogenesis of RA.[2,3]

Anti-TNF treatments

Biological agents such as antibodies and soluble TNF receptors that bind TNF α with high specificity neutralise its activity and have been developed for use as therapeutic agents. Several are currently being evaluated in patients with RA (table 1). Infliximab, cA2, is a chimeric monoclonal antibody (mAb) that consists of the variable region of a murine anti-TNF mAb coupled to the constant region of human IgG1κ.[4,5] The resulting construct is approximately two thirds human. CDP571 is a humanised mAb consisting of the complementarity determining regions of a murine anti-TNF mAb grafted into a human immunoglobulin (IgG4κ).[6] This mAb is approximately 95% human. Etanercept is a fusion protein consisting of two recombinant p75 TNF receptors attached to the Fc portion of a human IgG1.[7] Although this construct consists of two independent elements, which themselves contain 100% human peptide sequences, they are arranged in an unnatural configuration.

The duration of the therapeutic efficacy of these TNF antagonists may be limited by an immune response to their non-human elements or artificially fused human sequences. The development of antibodies to these biological agents could reduce their half life, thereby decreasing efficacy.[8] In addition, an immune response could result in adverse events from the formation of immune complexes or the development of hypersensitivity. For these reasons, an antibody that is fully human may have greater therapeutic potential.

Department of Clinical Oncology/Immunology, Knoll AG-BASF Pharma, Knollstrasse, D-67008 Ludwigshafen, Germany

Correspondence to:
Dr J Kempeni.

Table 1 Anti-TNF α treatments

Name	*Description*
D2E7	Fully human anti-TNF α mAb
CDP571	Humanised anti-TNF α mAb (murine CDR)
Etanercept	Fusion protein consisting of two recombinant p75 TNF α receptors and the Fc portion of human IgG1
Infliximab (cA2)	Chimeric murine/human anti-TNF-α mAb

TNF=tumour necrosis factor; mAb=monoclonal antibody; IgG=immunoglobulin G; CDR=complementarity determining region.

Development of a fully human anti-TNF antibody

BASF Pharma set out to develop a fully human, anti-TNF mAb structurally identical to naturally occurring human antibodies and therefore less likely to engender an immune response in the recipient. The generation of a fully human anti-TNF mAb required bioengineering techniques[9] that mimic immune selection in humans and contrast with existing means of "humanising" murine monoclonal antibodies in that the antibodies derived are completely human. The result of this effort is D2E7, a new class of anti-TNF mAb, which may have advantages in minimising antigenicity in humans.

Preclinical pharmacology

The efficacy and safety of D2E7 were evaluated in a number of experimental systems. The ability of D2E7 to neutralise TNF bioactivity was demonstrated in three different in vitro cell systems. Additionally, the effectiveness of D2E7 in preventing polyarthritis was shown in a transgenic mouse model that mimics the clinical and histopathological progression of RA in humans.[10] D2E7 treated mice showed no clinical signs of arthritis during the 11 week study period. Microscopical examinations of the ankle joints of the animals showed no histopathological changes. In contrast, control mice developed severe arthritis with cartilage destruction and bone erosion.

Preliminary clinical data

There are important similarities in the designs of the early clinical trials assessing D2E7 in RA. All studies enrolled patients with an established diagnosis of RA who also had active disease, as evidenced by having a combination of swollen and tender joints, increased concentrations of acute phase reactants, and prolonged early morning stiffness. In addition, all trials involved RA patients with long disease duration and a history of failure of several DMARDs. During the trials, patients were allowed to continue stable doses of NSAIDs and corticosteroids. Efficacy was assessed

using composite criteria, such as the American College of Rheumatology improvement criteria (ACR 20)[11] and the Disease Activity Score (DAS).[12] Such criteria, which require improvement in multiple variables, are more stringent than is improvement in one or only selected clinical variables. For example, to be classified as a responder according to ACR 20 criteria, patients must demonstrate: (1) greater than or equal to 20% improvement in swollen joint count; (2) greater than or equal to 20% improvement in tender joint count and; (3) at least 20% improvement in three of five other measures (patient global assessment of disease activity, physician global assessment of disease activity, patient assessment of pain, an acute phase reactant (for example, erythrocyte sedimentation rate (ESR) or C reactive protein), and a measure of disability (for example, the Health Assessment Questionnaire)). The DAS is a composite score of tender joints, swollen joints, ESR and patient's assessment of disease activity as measured on a visual analogue scale.

Preliminary results of early trials of D2E7 are available (table 2). At the time of this review, some of the studies referenced have only been published in abstract form. In a randomised, double blind, placebo controlled phase I study, 120 patients were treated with single doses of D2E7 given in an ascending fashion ranging from 0.5 to 10 mg/kg.[13] Patients were treated in cohorts of 24 (18 patients were treated with D2E7 and six received placebo). After a wash out period of three weeks, the patients received a single dose of D2E7 or placebo by intravenous injection over 3–5 minutes. Patients were followed up for at least four weeks to determine the pharmacokinetics of D2E7, as well as to evaluate the safety and clinical efficacy of the compound in terms of onset, duration and magnitude of response. Positive response was defined as a decrease of at least 1.2 (compared with baseline) in the DAS. All parameters needed to calculate the response criteria as defined by the ACR 20 were also measured. Patients in whom the effect of D2E7 had declined below response status by week 4 entered an open label extension study. However, patients who maintained a response at week 4 were continued without retreatment until their response status was lost. Thereafter, these patients could also continue in the extension study.

The data from this first therapeutic trial in humans were very encouraging. In the three highest dose groups, 40–70% of patients achieved DAS and ACR 20 response status at 24 hours to 29 days of treatment. The therapeutic effects became evident within 24 hours to one week after D2E7 administration and reached the maximum effect after 1–2 weeks, with dose response reaching a plateau at 1 mg/kg D2E7. In contrast, only 19% of patients taking placebo achieved response status. Single doses of D2E7 were well tolerated and the dose increment scheme was followed as planned reaching the maximum dose of 10 mg/kg without any evidence of clinically relevant or dose related adverse effects. Pharmacokinetic parameters were calculated for a total of 89 patients from all dose groups. The systemic drug exposure (AUC) increased proportionally with increased dose. The mean total serum clearance was 0.180 to 0.271 ml/min, and the steady state volume of distribution ranged from 0.063 to 0.076 l/kg indicating that distribution of D2E7 was mostly in the intravascular space. The estimated mean terminal half life was 11.6 to 13.7 days.

Patients who participated in the open label extension study received a second blinded dose identical to their first dose (medication was given after a minimum period of four weeks and only after loss of their initial response status).[14] From the third dose onwards, all patients were given active drug (that is, the placebo patients received D2E7 doses according to their dose group). D2E7 was administered every two weeks until responses could be rated as "good", defined as an absolute DAS of < 2.4. To measure the duration of the good response, these patients were retreated only upon disease flare up. To keep as many patients as possible in the study for the long term evaluation of safety, patients who did not respond well after 0.5 or 1 mg/kg received higher doses of up to a maximum of 3 mg/kg. Doses were kept constant in the patients on 3, 5 or 10 mg/kg. Treatment lasting several years is intended. The response in the DAS over time demonstrated sustained therapeutic effects and some continuing improvement after multiple infusions of D2E7. Response rates of more than 80% have been achieved with a mean dosing interval of 2.5 weeks. After six months, 86% of patients continued to receive treatment with D2E7 indicating that long term intravenous treatment with D2E7 in the dose range from 0.5 to 10 mg/kg was well tolerated.

The safety and efficacy of weekly subcutaneous administration of 0.5 mg/kg D2E7 was evaluated in 24 patients with active RA in a phase I placebo controlled trial.[15] After a wash

Table 2 Early trials of D2E7 in rheumatoid arthritis

Trial design	*Patients*	*D2E7 dosing schedule*	*Concurrent DMARD*	*Maximum ACR 20 response*
Randomised, double blind, placebo controlled	n=120; 83% RF⁺; mean disease duration=12 years; mean DMARDs failed=3.6	Single and multiple iv injections, ascending doses ranging from 0.5 to 10 mg/kg	No	78%
Randomised, double blind, placebo controlled	n=24; mean disease duration=10 years; mean DMARDs failed=3.4	Weekly 0.5 mg/kg sc injections	No	70%
Randomised, double blind, placebo controlled	n=34; 87% RF⁺; mean disease duration=11 years; mean DMARDs failed (including MTX)=3.6	Single iv or sc injection of 1 mg/kg	MTX	67%*

RF⁺=rheumatoid factor positive; DMARD=disease modifying anti-rheumatic drug; MTX= methotrexate; iv=intravenous; sc=subcutaneous. *With subcutaneous administration.

out period of three weeks, patients were treated with subcutaneous D2E7 or placebo for three months. The dose of D2E7 was increased to 1 mg/kg subcutaneously weekly for non-responders or those losing their responder status. Blood samples were collected to determine D2E7 plasma concentrations. All responding patients continued in an open label extension of this study. Based on preliminary data, plasma concentrations of D2E7 after multiple subcutaneous doses were comparable to those achieved with intravenous administration. Up to 78% of patients achieved a DAS/ACR 20 response after three months of treatment with subcutaneous D2E7. With the exception of mild and transient injection site reactions, adverse events occurred with the same frequency and distribution in the D2E7 and placebo groups. The investigators concluded that D2E7 given subcutaneously was safe and as effective as when administered intravenously demonstrating that subcutaneous self administration is a promising approach for D2E7 delivery.

Monotherapy is often inadequate to control arthritic symptoms and rapid progression of RA. D2E7 (1 mg/kg as a single subcutaneous or intravenous injection) was evaluated in a randomised, double blind, placebo controlled trial in patients whose stable dose of methotrexate was insufficient to control symptoms. An ACR 20 response was seen in 67% and 72% of patients receiving D2E7 by subcutaneous and intravenous injection, respectively. The safety profile of single dose D2E7 administration was comparable to that of placebo.

Collectively, these early data suggest that the fully human anti-TNFα mAb D2E7 is safe and effective as monotherapy or in combination with methotrexate when administered by single and multiple intravenous and subcutaneous injections. Additional studies are underway to further define optimal use of this novel treatment.

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Antibody Engineering: Research and Application of Genes Encoding Immunoglobulins

T 522 LIPID-TAGGED ANTIBODIES FOR IMMOBILIZATION ONTO LIPID BILAYERS, Marja-Leena Laukkanen and Kari Keinänen, VTT Biotechnical Laboratory, P.O.Box 202, SF-02151 Espoo, Finland

We have studied possibilities to anchor antibody fragments onto lipid bilayers via hydrophobic tags for immunodiagnostic and other applications. Previously we reported that a bacterially produced anti-2-phenyloxazolone single-chain Fv antibody fragment (Ox scFv, Takkinen *et al.*, 1991) can be converted by genetic engineering into a membrane-anchored protein by fusion with the major lipoprotein (lpp) of *E. coli* (Laukkanen *et al.*, 1993). The resulting antibody fragment (Ox lpp-scFv) contains an N-terminal covalently- bound lipid tag and is stably associated with bacterial cell envelope and displays antigen-binding activity in membrane-bound as well as detergent-solubilized form.
In order to purify the fusion protein by immobilized metal affinity chromatography (IMAC), a lipid-tagged antibody with poly-His tail (Ox lpp-scFv-H6) was constructed. The expression of the Ox lpp-scFv-H6 in *E. coli* resulted in the production of a 30 kDa fatty acid modified protein with antigen-binding profile similar to parental Ox lpp-scFv. Purification by IMAC followed by hapten affinity chromatography yielded a highly purified lipid-tagged antibody which was reconstituted into liposomes by detergent dialysis. Liposomes carrying the antibody show specific antigen-binding activity measured as by ELISA and by real-time biospecific interaction analysis using BIAcore.
Lipid-tagged antibodies may find use in immunoliposome, vaccine and biosensor technology.

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T 523 USE OF ALANINE SCANNING MUTAGENESIS TO IMPROVE THE AFFINITY OF AN ANTI gp120 (HIV) ANTIBODY
Craig M. Lewis, Jwu-Sheng Tung, George E. Mark, Greg F. Hollis and Steven W. Ludmerer, Department of Cellular and Molecular Biology, Merck Research Laboratories, Rahway, NJ 07065

Antibodies that recognize V3 loop peptides of HIV gp120 have been shown to neutralize HIV*in vitro* and *in vivo*. It has been demonstrated that the antibody-antigen off-rate is an important parameter in neutralization, therefore the ability to decrease the off-rate of an antibody through mutagenesis is likely to increase its therapeutic potential. We isolated several mutants of a single chain Fv antibody (clone P5Q) with reduced off-rates to V3 loop peptides. Critical residues of the VH CDR3 region were identified by alanine substitution mutagenesis (alanine scanning). Four classes of affinities, as determined by BIAcore analysis, were observed upon replacement of alanine at each of the 27 amino acid positions of CDR3: i) increased binding (2), ii) decreased binding (5), iii) no binding (6), and iv) no change (14) relative to P5Q. Positions that resulted in increased or reduced binding (classes i and ii), operationally defined as critical, were candidates for further study.

The two class i positions (improved off-rates) were subsequently randomized to all amino acids and optimal solutions determined. In one case the optimized improvement is observed with glutamic acid (2.8 fold improvement relative to P5Q). Smaller improvements are observed with other polar or negatively charged amino acids, while binding was eliminated with hydrophobic residue substitutions. In the second case optimized improvement was observed with tryptophan (4.7 fold), and measurable improvements were observed with several hydrophobic residues. Thus the method is not limited to just one kind of amino acid substitution. Additional studies on one of these positions demonstrates that the improvement is observed with several gp120 V3 loop peptide variants.

To date, one member of class ii (decreased binding) has been randomized. In contrast to the class i residues described above, the aspartic acid which appears at this position in P5Q is the optimal residue. To determine whether or not improvements are additive, scFv derivatives which contain optimal amino acids at some or all of these positions are being evaluated.

T 524 CONSTRUCTION OF SINGLE-CHAIN ANTIBODY FRAGMENTS AND EVALUATION IN WHOLE-BLOOD DIAGNOSTIC KITS, Glenn G. Lilley, Greg Coia, Olan Dolezal, Carmel Hillyard[1], Dennis Rylatt[1] and Peter Hudson, CSIRO Division of Biomolecular Engineering, 343 Royal Parade, Parkville, Australia, 3052, and [1]AGEN Biomedical Limited, P.O. Box 391, Acacia Ridge, Australia, 4110.

We have designed, constructed and expressed scFv molecules that can replace Fab fragments as red blood cell agglutination reagents. The new technology has enabled the construction of antibody-like molecules with multiple functionality or altered or even increased specificity.
The SimpliRED diagnostic kit for HIV-1 (AGEN Biomedical Ltd., Australia) incorporates as the active reagent, a bifunctional, antibody-based molecule which is constructed by protein chemical techniques from the Fab portion of a mouse monoclonal antibody and a synthetic peptide epitope. The Fab domain recognises the glycoprotein, glycophorin-A found on the surface of human erythrocytes and the linked peptide epitope is in turn recognised by antibodies in the sera of individuals who have contacted and raised an immune response to HIV. The addition of the reagent to the blood of these patients causes rapid red cell agglutination.
The monoclonal antibody which forms the basis of the SimpliRED kit reagent has been cloned and the functional variable domains have been sub-cloned into *E.coli* expression vectors in the form of an scFv molecule fused to the FLAG octapeptide epitope or alternatively to HIV-1 epitopes (gp41, gp120 and p24 fragments). The products expressed in *E.coli* are recognised in Western blots by monoclonal antibodies directed against the C-terminal epitopes. The recombinant fusion protein of scFv and FLAG epitope can mimic the commercial reagent in agglutination assays. Research is directed towards the improvement of the expression system and purification methods for the use of the recombinant scFv reagents in robust diagnostic kits.

T 525 IDIOTYPE MAPPING OF ANTI-DNA Fab FRAGMENTS EXPRESSED IN E. COLI,

Offen Daniel*, Irit Kinor * and Betty Diamond # Felsenstein Medical Center Beilinson Campus Petach-Tikva 49100 ISRAEL *, Albert Einstein College of Medicine New York, NY 10469 #

Patients with lupus erythematosus (SLE) characteristically produce antibodies to (ds)DNA. The pathogenic importance of these antibodies is suggested by their fluctuation with disease activity. Idiotypic analysis of anti-DNA antibodies have been informative regarding the structural basis for DNA binding and for pathogenicity. Serum titter of 31 and F4 idiotypes, identified by monoclonal antibodies, correlate with serum levels of anti-dsDNA activity in SLE patients. This study has focused on the 2A4 antibody which possesses high affinity anti-dsDNA activity and expresses both the 31 and F4 idiotypes, located on the light and heavy chains respectively. Using PCR we have amplified the 2A4 C_H1, V_H4 and C_k, V_k1 genes, 700bp each, and insert them separately or together into the pCOMB-3 plasmid vector. Several E.Coli clones expressing light chain, heavy chain and Fab fragments were obtained. High level expression can be seen both in bacteria cells and supernatants. Deletions and directed mutagenesis are been performed now in order to identify the exact idiotypes sequences. This antibody engineering approach provides a means for idiotype analysis and may identify the correlates to idiotypic and antigenic specificity and pathogenicity.